Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 808 (2004) 75–82
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Analysis of synthetic derivatives of peptide hormones by capillary zone electrophoresis and micellar electrokinetic chromatography with ultraviolet-absorption and laser-induced fluorescence detection

Veronika Šolínová, Václav Kašička*, Dušan Koval, Tomislav Barth, Alice Ciencialová, Lenka Žáková

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo 2, 166 10 Prague 6, Czech Republic

Available online 27 February 2004

Abstract

Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) were used for the analysis of new synthetic derivatives of hypophysis neurohormones—vasopressin and oxytocin, and pancreatic hormone—human insulin (HI) and its octapeptide fragment, derivatized by fluorescent probe, 4-chloro-7-nitrobenzo[1,2,5]oxadiazol (NBD). The suitable composition of background electrolytes (BGEs) was selected on the basis of calculated pH dependence of effective charge of analyzed peptides. Basic ionogenic peptides were analyzed by CZE in the acidic BGE composed of 100 mM H_3PO_4, 50 mM Tris, pH 2.25. The ionogenic peptides with fluorescent label, NBD, were analyzed in 0.5 M acetic acid, pH 2.5. The best MEKC separation of non-ionogenic peptides was achieved in alkaline BGE, 20 mM Tris, 5 mM H_3PO_4, with micellar pseudophase formed by 50 mM sodium dodecylsulfate (SDS), pH 8.8. Selected characteristics (noise, detectability of substance, sensitivity of detector) of the UV-absorption detectors (single wavelength detector, multiple-wavelength photodiode array detector (PDA), both of them operating at constant wavelength 206 nm) and laser-induced fluorescence (LIF) detector (excitation/emission wavelength 488/520 nm) were determined. The detectability of peptides in the single wavelength detector was 1.3–6.0 μmol dm^{-3} and in the PDA detector 1.6–3.1 μmol dm^{-3}. The LIF detection was more sensitive, the applied concentration of NBD derivative of insulin fragment in CZE analysis with LIF detection was three orders lower than in CZE with UV-absorption detector, and the detectability of this peptide was improved to 15.8 nmol dm^{-3}.

Keywords: Derivatization, CE; Derivatization, MEKC; Insulin; Peptides

1. Introduction

High-performance capillary electromigration methods, capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC), providing fast (few minutes) and high-efficient separation (10^5–10^6 theoretical plates per meter) of picomole to attomole quantities of analytes in the nanolitre sample volume, have a high application potential in the field of chemistry of peptides: they are broadly utilized for separation, analysis, preparation and characterization of peptides in chemistry, biochemistry, biomedicine, biotechnology, pharmacy and agriculture, as documented in several recent reviews [1–7].

Peptides and their analogs, fragments and derivatives are either isolated from the natural material or synthesized from amino acid building blocks. Sometimes they are prepared semisynthetically; i.e. synthetic peptide is attached to the isolated peptide or its fragment. In all cases it is important to check the success of the whole isolation or synthesis, eventually single steps of the process, because the obtained products can contain various admixtures, e.g. by-products of peptide synthesis. This is a suitable task for capillary electrophoresis (CE), since determination of purity degree is the most common application of CE in peptide chemistry [2].

One group of biopeptides analyzed in this work are represented by synthetic derivatives of neurohormones, oxytocin and vasopressin, produced by the pituitary gland (hypophysis) [8]. Oxytocin is responsible for contraction of the uterus in labor and ejection of milk from mammary glands during breast-feeding. Vasopressin and its analogs

* Corresponding author. Tel.: +420-220-183-239; fax: +420-220-183-592.
E-mail address: kasicka@uochb.cas.cz (V. Kašička).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.01.062

are also known as hormones with anti-diuretic activity [9]; they stimulate the retention of water by kidneys [10].

Structurally similar analogues of vasopressin and other biopeptides, enkephalins and dalargins, have been separated by CZE in acidic background electrolyte (BGE), 150 mM phosphoric acid, pH 1.8 [11,12]. Analogues of dalargin were analyzed by CZE in 0.5 M acetic acid, pH 2.5, and then separated in preparative scale by free-flow zone electrophoresis [13]. These peptides were also separated by MEKC in 20 mM tetraborate BGE with micellar pseudophase formed by sodium dodecylsulfate (SDS), pH 9.2 [11,12]. Oxytocin, vasopressin and other neurohypophyseal nonapeptides were determined by MEKC in four different micellar systems—with cationic, anionic, zwitterionic and neutral micellar constituents [14]. Chiral separations of dalargin analogs with D- and L-isomers of phenylalanine in the fourth position in peptide chain were performed in 50 mM phosphate buffer, pH 2.5 with 10 mM β-cyclodextrin [12]. Peptide neurohormones, derivatives of vasopressin, enkephalins and dalargin, and human insulin (HI) were analyzed also by different variants of HPLC, reverse-phase [11,12] and ion-exchange [15,16] HPLC.

Insulin is the peptide hormone produced by pancreatic β-cells that regulates the metabolism of carbohydrates, proteins and fatty acids [17]. Insulin molecule consists of 2 polypeptide chains, A and B, containing 21 and 30 amino acid residues, respectively. The two chains of insulin are connected by two disulfide bonds and the third disulfide bond is intra-chain bond in chain A. Its relative molar mass is $M_r = 5808$, the isoelectric point, pI, is 5.4–5.5.

CZE and HPLC have been frequently used for analysis of human insulin and its derivatives. Stable derivative of insulin with exo-3,6-epoxy-1,2,3,6-tetrahydrophtalic anhydride (ETPA) were identified by ion-exchange HPLC and CE [18]. CZE with UV detection was utilized for analysis of HI in CHES-acetate-acetonitrile BGE, pH 7–8 [19]. CE was also used to validate the HPLC assay of insulin as an analytical method [20]. Human insulin, proinsulin and intermediate forms were simultaneously quantified by CE in TAPS (*N*-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid), DETA (diethylenetriamine) and methanol [21]. CZE with fluorescence detection was used mainly for immunoassay of insulin. In some cases the native fluorescence of insulin was detected (excitation 275 nm, emission 305 nm) [22]. The analysis was performed in acidic BGE—50 mM citric acid, pH 2.3, or in alkaline BGE 20 mM Tricine, pH 8.5. Insulin with bonded fluorescent dye NN 382 was analyzed in 50 mM phosphate and 25 mM potassium sulfate, pH 7.0 with laser-induced fluorescence (LIF) detection in near infrared range [23].

The aim of this work was to analyze synthetic derivatives and analogs of hypophyseal neurohormones, oxytocin and vasopressin, and of pancreatic hormone, human insulin, and its octapeptide fragment, B23-30-HI, derivatized by fluorescent label, 4-chloro-7-nitrobenzo[1,2,5]oxadiazol (NBD), by CZE and MEKC, in the home-made CE device with single wavelength UV-absorption detector and in commercial Beckman–Coulter CE-MDQ apparatus with multiple-wavelength UV-absorption detector and LIF detector. The rational approach, based on the calculated pH dependence of effective charge of peptides, is used for selection of suitable separation conditions. The sensitivity of UV and LIF detectors and detectability of non-labeled and labeled peptides as well as other characteristics of detectors are compared and discussed.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade. Tris (tris(hydroxymethyl)aminomethane) was obtained from Serva (Heidelberg, Germany), phosphoric acid, acetic acid and dimethyl sulfoxide (DMSO) were supplied by Lachema (Brno, Czech Republic) and sodium dodecylsulfate was from Fluka (Buchs, Switzerland).

2.2. Peptides

The sequences of analyzed peptides and their abbreviations are presented in Table 1. Analogs of oxytocin, vasopressin and octapeptide B23-B30 of the C-terminal part of the B-chain of human insulin (HI) were synthesized in our institute (IOCB) by the solid phase method described elsewhere [24]. 4-Chloro-7-nitrobenzo[1,2,5]oxadiazol (NBD) was used as fluorescent probe for B23-B30 octapeptide of HI. NBD reacted with free ϵ-amino group of lysine B29 in octapeptide of HI. The α-amino group of lysine was protected by the Fmoc protecting group [25]. Fluorescent-labeled human insulin was prepared semisynthetically by the trypsin-catalyzed peptide bond formation between C-terminal arginine B22 of desoctapeptide of insulin and N-terminal glycine residue of NBD-labeled octapeptide B23-B30 of HI [26].

2.3. Instrumentation

The electrophoretic experiments were carried out in two apparatuses. The first one was home made CE device developed in our institute, IOCB, further indicated as CE-IOCB [27]. It is equipped with single wavelength UV-absorption photometric detector operating at 206 nm; the light source is electrodeless, high-frequency excited iodine discharge lamp (LKB-Pharmacia, Uppsala, Sweden), and UV-sensitive Si-photodiode with built-in preamplifier (Hamamatsu Photonics Deutschland, Herrsching, Germany) is used as light detector. The bare fused silica capillary with outer polyimide coating, 50 μm i.d. × 200 μm O.D., total length 30 cm and effective length 19 cm, was supplied by the Institute of Glass and Ceramics Materials, Czech Academy of Sciences (Prague, Czech Republic). The separations were

Table 1
Sequences of analyzed peptides

Peptide	Abbreviation	Sequence in three-letters code	Sequence in single-letter code
Oxytocin	OT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH_2	CYIQNCPLG-NH_2
Deamino-oxytocin	dOT	Mpa-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH_2	MpaYIQNCPLG-NH_2
Arg^8-vasopressin	AVP	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH_2	CYFQNCPRG-NH_2
(Deamino-D-Arg^8)-vasopressin	dDAVP	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-D-Arg-GlyNH_2	MpaYFQNCPrG-NH_2
Insect diuretic hormone	IDH	Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-GlyNH_2	CLITNCPRG-NH_2
(Deamino-D-Arg^8)-IDH	dDA-IDH	Mpa-Leu-Ile-Thr-Asn-Cys-Pro-D-Arg-GlyNH_2	MpaLITNCPrG-NH_2
(D-Arg^8)-IDH	DA-IDH	Cys-Leu-Ile-Thr-Asn-Cys-Pro-D-Arg-GlyNH_2	CLITNCPrG-NH_2
(Deamino-Arg^8)-vasopressin	dAVP	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH_2	MpaYFQNCPRG-NH_2
NBD-derivative of B23-B30-human insulin fragment	NBD-B23-30-HI	Gly-Phe-Phe-Tyr-Thr-Pro-Lys-(NBD)-Thr	GFFYTPK(NBD)T

NBD: 4-chloro-7-nitrobenzo[1,2,5]oxadiazol.

performed at ambient temperature 22–24 °C without active cooling of the capillary.

The second apparatus was commercial P/ACE MDQ System (Beckman–Coulter, Fullerton, CA, USA), further indicated as CE-MDQ, with multiwavelength spectrophotometric photodiode array (PDA) detector (190–600 nm) and Ar-ion laser-induced fluorescence (LIF) detector (excitation wavelength 488 nm, emission wavelength 520 nm). The fused silica capillary with outer polyimide coating as well as in IOCB device was internally uncoated, total length was 40 cm and effective length was 30.2 cm, with diameters 75 μm I.D. × 360 μm O.D. 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 in the range 10–20 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 0.005–1.3 mg/ml for UV-absorption detection and 10–1000× lower for CE with LIF detection. 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 of the rational selection of conditions in CE separation of peptides follows the general rules of selection of suitable CE separation conditions [28,29] and takes into account the specific peptide properties. The specific properties of peptides result from their primary structure—number and sequence of the linked amino acid residues. The structure determines the electric charge, size (relative molecular mass), shape (conformation), hydrophobicity and specific binding capability. From the structure of peptides also their ionogenicity is deduced, which determines whether CZE or MEKC can be used for analysis of given peptide sample. CZE is used for analysis of ionogenic peptides and the separation is based on differences in electrophoretic mobilities, which are determined by charge, size and shape of peptide molecules. MEKC is used for analysis of non-ionogenic peptides; the separation is based on their different hydrophobicity.

The selection of the composition of the BGEs includes buffer components type and concentration, pH and takes into

account the request for chemical and temperature stability and biological activity of analyzed peptides [30]. Effective charges of peptides are strongly dependent on pH and pK_a of ionogenic groups of amino acid residues present in peptide chain. Analyzed peptides contain ionogenic groups—all except one α-NH_3^+ group of N-terminal of peptide chain (average pK_a 8.0), four of them guanidium of arginine (average pK_a 11.2) or hydroxy group of tyrosine (average pK_a 10.0) and form cyclic structures by disulfide bridges of cystine. The relative molecular mass of nonapeptides was around 1000. The pH dependence of effective charge was calculated with the earlier developed computer program Nabamfo [31] using the above given average values of pK_a of ionogenic groups. The program is based on mathematical model of acid–base equilibrium of general ampholyte. The effective charges of ionogenic peptides reached values +1 or +2 elementary charges in the whole acidic pH range. Some peptides behaved as weakly negatively charged in strongly alkaline BGE, which was not suitable for these peptides due to the lability of cystine bond at high pH. In conclusion, ionogenic peptides were analyzed by CZE as cations in acidic BGE. The pH dependence of the effective charges of analyzed nonapeptides is depicted in Fig. 1.

The solubility of peptides is given by their structure. The analyzed peptides were dissolved in deionized water or BGEs. Solubilization in deionized water is advantageous due to the possibility to apply the same sample solution for CE separations in different BGEs and in addition the concentrating effect is utilized. On the other hand using BGE as sample solvent ensures the constant separation conditions (BGE composition) in the whole CE experiment.

Ionogenic peptides were analyzed by CZE as cations in acidic BGEs. The best results were obtained in BGE composed of 100 mM phosphoric acid, 50 mM Tris, pH 2.25, and with the applied voltage 10 kV. The examples of peptide CZE analyses are shown in Fig. 2. The effective electrophoretic mobilities of analyzed peptides in the above tris-phosphate BGE are presented in Table 2. The average electroosmotic mobility of the tris-phosphate buffer was 3.46×10^{-9} m^2 V^{-1} s^{-1}. Peptides with two positive elementary charges migrated the fastest. Peptides containing tyrosine in the second position in peptide chain migrated slower than peptides with other amino acids in this position and the same total positive charge, obviously due to larger size of their hydrated molecules. On the other hand peptide containing arginine residue in position 8 instead of leucine migrated faster than peptides with free N-terminal group, the slowest migrating positively charged peptide had just free N-terminal group. The peptides with fluorescent label NBD were analyzed by CZE in 0.5 M acetic acid, pH 2.5, and with the applied voltage 15 kV. The examples of their electrophoregrams are shown in Fig. 3.

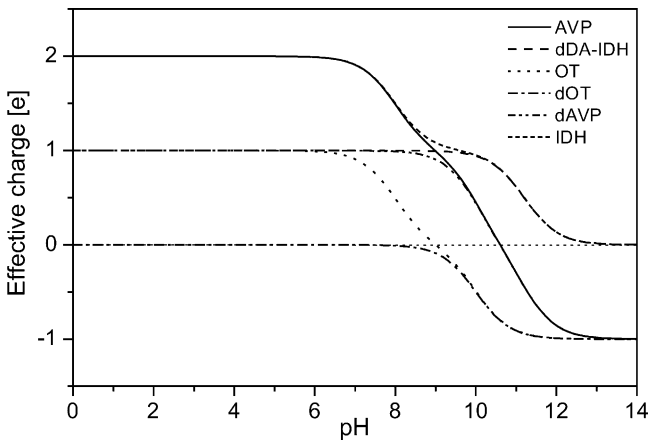

Fig. 1. The pH dependence of the effective charge of analyzed neurohypophyseal peptides. For peptide abbreviations see Table 1.

Table 2
Effective electrophoretic mobility and differently expressed purity degrees of peptides determined by CZE in home-made CE-IOCB device and in commercial CE-MDQ device with UV-absorption detection (206 nm) in BGE composed of 100 mM H_3PO_4, 50 mM Tris, pH 2.25

Peptide	m_{ef} (10^{-9} m^2 V^{-1} s^{-1})	P_h (%)	P_A (%)	P_{CA} (%)
Analyses in CE-IOCB device				
OT	11.6	95.6	95.0	94.5
dOT	0	93.3*	89.1*	89.3*
AVP	23.3	94.1	90.1	91.5
dDAVP	12.7	95.1	96.9	96.4
IDH	24.5	42.3	28.6	26.7
dDA-IDH	13.4	66.7	69.3	68.4
dAVP	12.6	92.0	89.9	89.3
DA-IDH	24.4	70.8	52.9	48.2
Analyses in CE-MDQ device				
OT	9.1	93.5	90.6	90.4
AVP	16.8	87.7	83.2	80.4

m_{ef}: Effective electrophoretic mobility; P_h, P_A, P_{CA}: purity degrees expressed on the basis of relative peak height, relative peak area and relative peak corrected area, respectively; (*): purity degrees of dOT obtained from MEKC analyses.

Non-ionogenic peptides with blocked or derivatized ionogenic groups were analyzed by MEKC in alkaline BGE—20 mM Tris, 5 mM H_3PO_4, pH 8.8, with micellar pseudophase formed by an anionic detergent sodium dodecylsulfate, see Fig. 4. The applied voltage was 20 kV. In all systems symmetrical peaks were obtained without any shoulders and with high separation efficiency in short times. All analyses did not take more than 7 min. The number of theoretical plates/meter was in the range 8×10^5 to 3×10^6.

3.2. Determination of purity degree

The purity degrees of the crude synthetic or semisynthetic peptide preparations after HPLC purification were determined. Standards of synthesized peptides were not available. Peptide purity and peptide content in the sample can be quantified by several ways: (i) relative peak height, $P_h(i)$ (1), (ii) relative peak area, $P_A(i)$ (2), and (iii) relative peak corrected area, P_{CA}(i) (3), of the UV-positive peaks

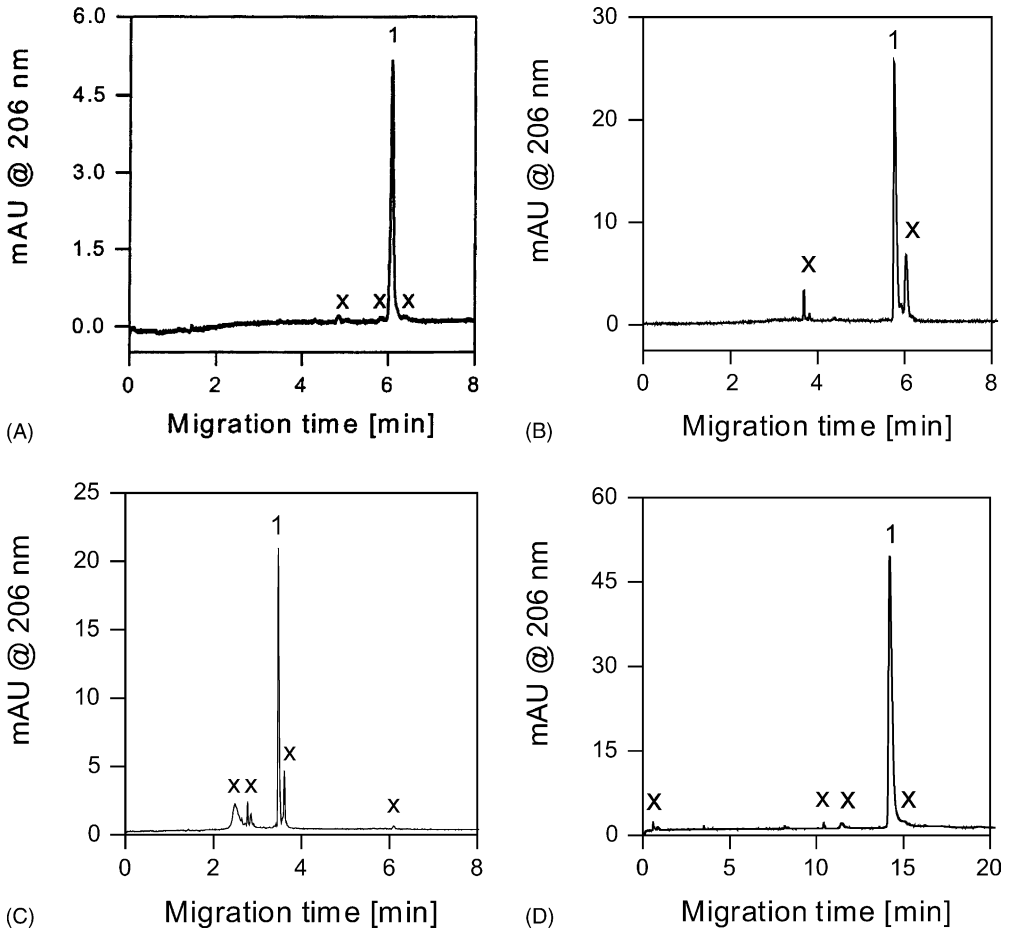

Fig. 2. CZE analyses of derivatives of hypophyseal neurohormones: (A) (deamino-Arg^8)-vasopressin, 0.40 mg ml^{-1}; (B) (deamino-D-Arg^8)-IDH, 1.15 mg ml^{-1}; (C) (D-Arg^8)-IDH, 1.0 mg ml^{-1}; all three analyses performed in CE-IOCB device, current 48–50 μA, injection 10 mBar × 5 s, (D) oxytocin, 0.95 mg ml^{-1}, in CE-MDQ device, 69–70 μA, injection 7 mBar × 5 s. All analyses performed in BGE composed of 100 mM H_3PO_4, 50 mM Tris, pH 2.25; applied voltage 10 kV; (1) main synthetic product; (×) non-identified admixtures.

for i-th component:

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

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

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

where $h(i)$ is height of the i-th peak, $A(i)$ is area of the i-th peak and $A_c(i)$ is corrected area of the i-th peak, corrected peak area is peak area corrected with respect to migration velocity of the given peak, i.e. it is equal to peak area divided by migration time of the given peak, n is the number of sample components.

The values of differently expressed purity degrees are presented in the Table 2. The values of purity degrees were determined as averages of two subsequent analyses, which differed less than 1%. Analyses of peptides with high purity degree are demonstrated by CZE of (deamino-Arg^8)-vasopressin in Fig. 2A and oxytocin in Fig. 2D. On the other hand analysis of peptide with low purity degree and with at least three major impurities is shown in electrophoregram of (deamino-D-Arg^8)-insect diuretic hormone in Fig. 2B. 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 velocities are not too different. The comparison of differently expressed purity degree is demonstrated on (D-Arg^8)-insect diuretic hormone in Fig. 2C. DA-IDH relative peak height differed in around 20% against relative peak area and relative peak corrected area. As follows from the presented data, the determination of purity degree depends on separation conditions and mostly also on the way of its determination.

3.3. Sensitivity of detection

3.3.1. UV-absorption detection

The most common detection of peptides in CE is UV-absorption detection in the range 200–220 nm, due to

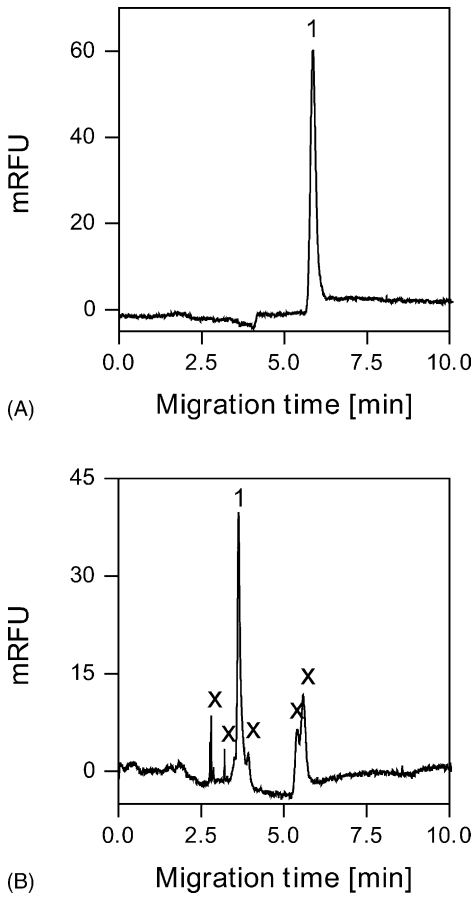

Fig. 3. CZE analyses of fluorescence labeled peptides in CE-MDQ device with LIF-detection: (A) NBD-B23-30-HI, 0.05 mg ml^{-1}; (B) NBD-HI, 0.5 μg ml^{-1}. Analyses performed in BGE composed of 0.5 M AcOH, pH 2.5; injection 10 mBar × 5 s; applied voltage 15 kV; current 20–21 μA; (1) main synthetic product; (×) non-identified admixtures.

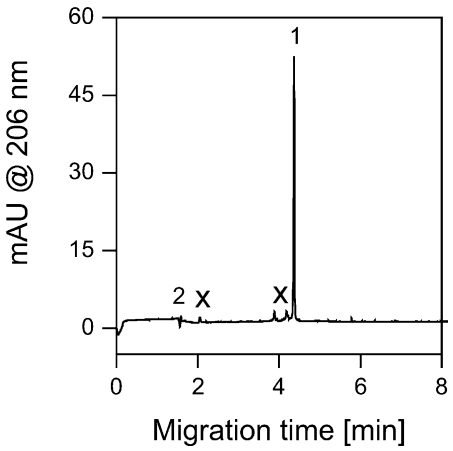

Fig. 4. MEKC analysis of non-ionogenic peptide, deamino-oxytocin, 1.15 mg ml^{-1}, in CE-MDQ device with PDA detector, BGE: 20 mM Tris, 5 mM H_3PO_4, 50 mM SDS, pH 8.8, injection 5 mBar × 10 s, applied voltage 20 kV, current 69–71 μA, (1) main synthetic product, (2) peak of electroosmotic flow marker, (×) non-identified admixtures.

the absorption of peptide bond CO–NH in this short wavelength UV region. The background electrolytes (BGEs) should not absorb light significantly in this range in order to prevent the decrease of the signal of analytes [7].

In this work two different UV-absorption detectors were tested. The comparison was performed from the analyses on new synthesized nonapeptides by CZE in acidic tris-phosphate buffer, pH 2.25. The first absorption detector used high-frequency excited iodine discharge lamp with emission at 206 nm and UV-sensitive silicon photodiode as detector of radiation. The second one was based on multiple-wavelength detection realized by photodiode array and deuterium lamp. Both detectors operated at constant wavelength 206 nm. The characteristics of the detectors, noise, n, response of the detector, R, signal to noise ratio, R/n, detectability of substance, D, and sensitivity of detector, S, were compared. The sensitivity of detector was calculated as the ratio of response of detector and concentration of analyte, $S = R/c$. The detectability of substance was determined as treble of noise divided by sensitivity, $D = 3n/S$. The values of the above characteristics of single wavelength detector (CE-IOCB) measured with oxytocin and Arg^8-vasopressin are presented in Table 3 and for PDA detector in CE-MDQ device in Table 4. The values were determined as averages of two subsequent analyses with maximal difference 2% in response of the detector. The ratio R/n of the oxytocin and Arg^8-vasopressin was slightly higher in PDA detector. The detectability of substances copied with small deviations the increase of R/n in PDA detector.

The influence of BGEs absorption on detector sensitivity and detectability of substance was investigated by CZE of oxytocin and Arg^8-vasopressin, performed in tris-phosphate buffer and acetic acid-based BGEs, respectively.

Table 3
The characteristics of single wavelength (206 nm) UV-absorption detector of CE-IOCB device determined from CZE analyses of peptides in BGE composed of 100 mM H_3PO_4, 50 mM Tris, pH 2.25

Peptide	c (μmol dm^{-3})	R (mV)	R/n	S (V mol^{-1} dm^3)	D (μmol dm^{-3})
OT	89.1	64.0	89.0	717.7	3.00
AVP	84.4	141.8	197.3	1680.6	1.28
dDAVP	1036	658.5	916.4	635.4	3.39
IDH	329	181.8	253.0	553.5	3.89
dDA-IDH	818	300.9	418.7	367.7	5.86

c: Peptide concentration; R: detector response; n: noise, average value of noise was 0.72 mV; S: sensitivity of detector, $S = R/c$; D: detectability of substance, $D = 3n/S$.

Table 4
The characteristics of the UV-absorption PDA detector (operating at 206 nm) of CE-MDQ device determined from CZE analyses of peptides in different BGEs, BGE I: 100 mM H_3PO_4, 50 mM Tris, pH 2.25; BGE II: 0.5 M acetic acid, pH 2.5

Peptide	BGE	c (mmol dm^{-3})	R (mAU)	R/n	S (AU.mol^{-1} dm^3)	D (μmol dm^{-3})
OT	I	0.85	43.8	1093.2	51.4	2.34
	II	0.91	16.4	859.2	18.0	3.18
AVP	I	0.92	67.2	1675.6	72.9	1.65
	II	0.86	24.6	1285.8	28.4	2.02

c: Peptide concentration; R: detector response; n: noise, average value of noise was 0.04 mAU for BGE I and 0.02 mAU for BGE II; S: sensitivity of detector, $S = R/c$; D: detectability of substance, $D = 3n/S$.

Table 5
The characteristics of the UV-absorption PDA detector set to 206 nm and LIF detector (488 nm/520 nm) of CE-MDQ device determined from CZE analyses of peptides in BGE composed of 0.5 M acetic acid, pH 2.5

Peptide	c (mmol dm^{-3})	R (mAU)	R/n	S (AU.mol^{-1} dm^3)	D (μmol dm^{-3})
Analyses with UV detector					
NBD-HI	629	2.77	184.7	44.1	1.02
NBD-B23-30-HI	72.9	8.57	571.1	11.7	3.83
Peptide	c (μmol dm^{-3})	R (mRFU)	R/n	S (RFU.mol^{-1} dm^3)	D (μmol dm^{-3})
Analyses with LIF detector					
NBD-HI	5.09	505.7	136.6	99331	0.112
NBD-B23-30-HI	0.89	629.1	169.9	706590	0.016

c: Peptide concentration; R: detector response; n: noise, average value of noise was 3.70 mRFU for LIF detector and 0.02 mAU for PDA detector; S: sensitivity of detector, $S = R/c$; D: detectability of substance, $D = 3n/S$.

The acetic acid absorbs more light, the peaks were smaller and noise was also lower. The value of the noise in tris-phosphate buffer was 0.0401 mAU and in acetic acid reached 0.0191 mAU in Beckman CE-MDQ with PDA detector. The separation in acetic acid BGE was influenced by electromigration dispersion; the peaks were wide and non-symmetrical. The sensitivity of PDA detector decreased 2.5 times in acetic acid BGE; the detectability was lower in comparison with tris-phosphate buffer in both equipments. On the other hand, the differences were not dramatic (see Table 4) and acetic acid can be also used as BGE in CZE.

3.3.2. LIF detection

Laser-induced fluorescence detection is the most sensitive detection in CE, its sensitivity is two or three orders higher than UV-absorption detection. On the other hand it is necessary to derivatize peptides by fluorogenic labels. Only peptides with aromatic amino acid residues of tryptophane and tyrosine exhibit native fluorescence in the region 200–300 nm. The necessary condition for excitation is UV-laser systems or multiphoton excitation [7].

In this work, the sensitivity of PDA and LIF detectors of CE-MDQ device were compared. Firstly, the derivatives of human insulin and its B23-30 octapeptide fragment with NBD label, NBD-HI and NBD-B23-30-HI, respectively, were detected by PDA detector in CE-MDQ device in 0.5 M acetic acid, pH 2.5. Fluorescent probe NBD was bonded on ϵ-amino group of lysine B29, the excitation maximum was at 475–478 nm and the emission maximum at 530–534 nm in pH 3–8 [32]. Labeled human insulin is necessary for monitoring of interactions between insulin and insulin-receptor at low concentrations in in vivo conditions. Consequently, LIF detector with Ar-ion laser (excitation at 488 nm, emission at 520 nm) was used for analyses of NBD-derivatives of HI and its fragment. The electrophoregrams of peptide analyses in acetic acid, pH 2.5, with LIF detection are depicted in Fig. 3. Whereas single peak was obtained for NBD-derivative of B23-30 octapeptide fragment of human insulin (see Fig. 3A), several admixtures have been found by CZE analysis of NBD-labeled human insulin, as shown in Fig. 3B. This indicates that the rest of fluorescent probe was not completely removed from the octapeptide fragment and it reacted with non-protected groups of amino acid residues of desoctapeptide of HI. For LIF detection the concentration of human insulin was 10 times lower than for UV-absorption detection, for octapeptide the solution was diluted 1000 times due to too high signal in LIF detector. The main characteristics of detectors as well as the comparison of UV detectors are shown in Table 5. The detectability of substance is one or two orders better for fluorescence detection.

4. Conclusions

CZE and MEKC proved to be powerful and useful tools for fast and sensitive analyses of ionogenic or non-ionogenic derivatives of peptide hormones, oxytocin, vasopressin,

human insulin and its fragment. The purity degrees and effective mobilities of ionogenic peptides were determined by CZE in acidic BGEs. Purity degree of non-ionogenic peptides was evaluated by MEKC in alkaline BGE with SDS micellar pseudophase. Sensitivity of single wavelength UV-absorption detector in home-made CE device was found to be comparable with that of multiple-wavelength PDA detector in commercial Beckman CE-MDQ apparatus. LIF detector was able to detect NBD-labeled octapeptide fragment of human insulin with two orders lower detectability than UV-absorption detector.

Acknowledgements

We wish to acknowledge the financial support from the Grant Agency of the Czech Republic, grants no. 203/02/1467, 203/03/0716, and from the Czech Academy of Sciences, research project no. AVOZ4055 905. Mrs. V. Lišková is thanked for her skilful technical assistance and other collegues from our laboratory, Dr. P. Sázelová and Dr. Z. Prusík, for their help in preparation of this publication.

References

[1] P.G. Righetti, Biopharm. Drug Dispos. 22 (2001) 337.
[2] V. Kašička, Electrophoresis 22 (2001) 4139.
[3] H.J. Issaq, T.P. Conrads, G.M. Janini, T.D. Veenstra, Electrophoresis 23 (2002) 3048.
[4] M.T.W. Hearn, Biologicals 29 (2001) 159.
[5] S. Hu, N.J. Dovichi, Anal. Chem. 74 (2002) 2833.
[6] Z. Deyl, I. Mikšík, in: Z. Deyl (Ed.), Advanced Chromatographic and Electromigration Methods in BioSciences, Elsevier, Amsterdam, 1998, p. 465.
[7] V. Kašička, in: H.Y. Aboul-Enein (Ed.), Analytical and Preparative Separation Methods of Biomacromolecules, Marcel Dekker Inc., New York, 1999, p. 39.
[8] O. Hanč, Z. Pádr, Hormony, Academia, Praha, 1982, p. 274.
[9] N. Sewald, H.D. Jakubke, in: N. Sewald, H.D. Jakubke (Eds.), Peptides: Chemistry and Biology, Wiley-VCH Verlag, Weinheim, 2002, p. 61.
[10] J. Mařík, M. Buděšínský, J. Slaninová, J. Hlaváèek, Collect. Czech. Chem. Commun. 67 (2002) 373.
[11] J. Jiskra, V. Pacáková, M. Tichá, K. Štulík, T. Barth, J. Chromatogr. A 761 (1997) 285.
[12] V. Pacáková, J. Suchánková, K. Štulík, J. Chromatogr. B. 681 (1996) 69.
[13] V. Kašička, Z. Prusík, J. Pospíšek, J. Chromatogr. 608 (1992) 13.
[14] N. Sutcliffe, P.H. Corran, J. Chromatogr. 636 (1993) 95.
[15] G. Mandrup, J. Chromatogr. 604 (1992) 267.
[16] J. Frenz, S.L. Wu, W.S. Hancock, J. Chromatogr. 480 (1989) 379.
[17] K. Huml, T. Barth, Chem. Listy 92 (1998) 294.
[18] K. Zaitsu, Y. Kimura, Y. Ohkura, Biol. Pharm. Bull. 17 (1994) 763.
[19] A. Kunkel, S. Günter, C. Dette, H. Wätzig, J. Chromatogr. A. 781 (1997) 445.
[20] A. Kunkel, S. Günter, H. Wätzig, Am. Lab. 30 (1998) C76.
[21] C. Arcelloni, L. Falqui, S. Martinenghi, A.E. Pontiroli, R. Paroni, Electrophoresis 19 (1998) 1475.
[22] W. Tong, E.S. Yeung, J. Chromatogr. B Bio. Med. Appl. 685 (1996) 35.
[23] J. Sowell, R. Parihar, G. Patonay, J. Chromatogr. B 752 (2001) 1.
[24] N. Sewald, H.D. Jakubke, in: N. Sewald, H.D. Jakubke (Eds.), Peptides: Chemistry and Biology, Wiley-VCH Verlag, Weinheim, 2002, p. 135.
[25] A. Ciencialová, L. Klasová, J. Jiráček, J. Barthová, T. Barth, in: J. Slaninová (Ed.), Biological Active Peptides, Collection Symposium Series, 4, Praha, 2001, p. 41.
[26] A. Ciencialová, L. Klasová, J. Barthová, T. Barth, Book of Abstracts “Biological Active Peptides”, IOCB, Praha, 2003, p. 17.
[27] V. Kašička, Z. Prusík, P. Sázelová, E. Brynda, J. Stejskal, Electrophoresis 20 (1999) 2484.
[28] P.G. Righetti, Capillary Electrophoresis in Analytical Biotechnology, CRC Series in Analytical Biotechnology, CRC Press, Boca Raton, 1996, p. 39.
[29] H.J. Issaq, G.M. Janini, K.C. Chan, Z. El Rassi, in: P.R. Brown, E. Grushka (Eds.), Advances in Chromatography, vol. 35, Marcel Dekker Inc., New York, 1995, p. 101.
[30] G.M. Janini, H.J. Issaq, Chromatographia 53 (2001) S18.
[31] V. Kašička, Z. Prusík, J. Chromatogr. 470 (1989) 209.
[32] A. Ciencialová, Graduation Report, Faculty of Natural Sciences, Charles University, Praha, 2003.