Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 841 (2006) 145–151
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of purity degree and counter-ion content in lecirelin by capillary zone electrophoresis and capillary isotachophoresis☆

Petra Sázelová, Václav Kašička*, Veronika Šolínová, Dušan Koval

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

Received 19 January 2006; accepted 7 April 2006
Available online 9 May 2006

Abstract

Capillary zone electrophoresis (CZE) and capillary isotachophoresis (CITP) were applied for the determination of peptide purity degree and counter-ion content in lecirelin, the synthetic analogue of luteinizing hormone-releasing hormone (LHRH). CZE analyses were carried out in acidic background electrolyte (100 mM H_3PO_4, 50 mM Tris, pH 2.25) in bare fused silica capillary using UV-absorption detection at 206 nm. CITP analyses were performed in the electrophoretic analyzer with column coupling, equipped with contactless conductivity detectors both in preseparation capillary and in analytical capillary, and with UV-absorption detector (220 and 254 nm) in analytical capillary. Determinations of peptide purity were carried out in cationic mode with leading electrolyte (LE), 10 mM KOH/AcOH, pH 4.5, and terminating electrolyte (TE), 10 mM β-alanine (BALA)/AcOH, pH 4.4. Degree of peptide purity determined by both CZE and CITP was in the range 60.1–80.9% for crude preparations of lecirelin and in the range 96.4–99.9% for HPLC purified batches. Concentrations of contaminating counter-ions, the anions of trifluoromethanesulfonic acid (TFMSA), trifluoroacetic acid (TFA) and acetic acid (AcOH), were determined by CITP analyses in anionic mode with LE 10 mM HCl/His, pH 6.0, and TE 10 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 4.0, by the calibration curve method. Mass percentages of the counterion contents in the analyzed lecirelin batches varied from zero to ca. 9% (TFMSA), 3% (TFA) and 11% (AcOH), respectively.

Keywords: Capillary zone electrophoresis; Capillary isotachophoresis; Lecirelin; Trifluoromethanesulfonic acid; Trifluoroacetic acid; Counterion content

1. Introduction

Lecirelin is a synthetic hormone, a strongly basic nonapeptide with sequence pGlu-His-Trp-Ser-Tyr-D-Tle-Leu-Arg-Pro-NH-Et. It is an analogue of hypothalamic luteinizing hormone-releasing hormone (LHRH), decapeptide with sequence pGlu-Ile-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, which stimulates both pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). It was shown, that the analogues with replaced Gly in the position 6 by other amino acids are more active than the natural peptide, e.g. [D-Ala^6]-LHRH exhibits 350–400% of activity of natural hormone [1]. Moreover, nonapeptide [des-GlyNH_2^{10} Pro-NHEt^9]-LHRH is 2–2.5 times more active than the natural decapeptide LHRH [2].

Lecirelin, shortly indicated as [D-Tle^6, Pro-NHEt^9]-LHRH, was first synthesized in 1983 by Flegel et al. [3]. These authors have found out that this LHRH analogue with D-*tert*-leucine (D-Tle) in the position 6 is significantly more active in sustaining enhanced LH and FSH in heifers in comparison with [D-Ala^6, Pro-NHEt^9]-LHRH (kobarelin) and natural LHRH.

Lecirelin has been registered as a veterinary drug Supergestran (Ferring-Léčiva, Prague, CR). The drug is intended for the induction of ovulation in livestock, both for treatment of ovarian cysts and for improvement of conception rates. It is used, e.g. for synchronization of oestrus and timing of ovulation in cows [4] and buffalo [5]. Moreover, lecirelin, kobarelin and other LHRH analogues are used in aquaculture for artificial spawning of various species of fishes in combination with dopamine receptor antagonists, e.g. pimozide, domperidone or metoclopramide, which potentiate the response to the peptide (so called Linpe method) [6]. The preparations containing metoclopramide

☆ This paper is part of a special volume entitled "Analysis of proteins, peptides and glycanes by capillary (electromigration) techniques", dedicated to Zdenek Deyl, guest edited by I. Miksik.

* 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 © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.04.006

together with lecirelin or kobarelin are known as Dagin and Ovopel, respectively. They were used for artificial spawning of carp [7,8], pike [9] and African and European catfish [10]; treating concentrations of lecirelin or kobarelin and metoclopramide used to be 10–20 μg/kg and 10–20 mg/kg body mass, respectively.

Synthetic (artificial) peptides are mostly synthesized by the solid phase method (solid-phase peptide synthesis, SPPS); now fully automated. Resulting peptide preparations may contain closely related peptide impurities, which originate from the side or incomplete reactions. For the peptide purification, preparative RP-HPLC method is frequently used, and then the peptides ought to be checked for purity by analytical RP-HPLC method and/or more preferentially by other separation techniques, based on different separation principles, such as capillary zone electrophoresis (CZE) or capillary isotachophoresis (CITP) [11]. Complementarity of HPLC and capillary electrophoretic (CE) methods was well demonstrated by a comparison of HPLC and CE analyses of synthetic biologically active peptides, e.g. bradykinin [12], enkephalins and dalargin [13]. Although CZE is more frequently used for the determination of purity degree of synthetic peptides than CITP [11], the role of CITP in the analysis of peptides is also important [14]. For the first time CITP was applied for peptide analysis in 1974 by Kopwillem et al. who analyzed the fragments of human growth hormone [15]. Since that time CITP has been used for analysis of many other naturally occurring and/or synthesized biologically active peptides [16,17]. Application of both HPLC and CITP for the control of the peptide purity of insulin, adrenocorticotropic hormone (ACTH), β-endorphin and other peptides and the complementary nature of the information obtained from both the methods were demonstrated by Janssen et al. [18]. The usage of CZE or CITP in peptide analysis in addition to HPLC gives more reliable information about peptide purity that is very important especially in the case of the peptides synthesized for therapeutic purposes, both in human and veterinary medicine.

At the end of lecirelin synthesis, trifluoromethanesulfonic acid (TFMSA) in trifluoroacetic acid (TFA) is used for splitting off the tosyl group protecting the guanidinyl group of arginine. Then the peptide is purified by RP-HPLC, using mobile phase containing TFA as well. Basic peptides including lecirelin are commonly isolated upon lyophilization in the form of salts, such as acetates or chlorides. In addition to these two physiologically tolerable anions, the toxic anions of the above mentioned acids (TFA, TFMSA) used in the synthesis and/or purification procedure of lecirelin may occur in the preparations of this peptide. Due to their toxicity or because they may reduce the biological potency of the peptide, they have to be removed from the peptide prior to preparation of the final formulation. Therefore, complete analysis of the synthesized peptide has to include determination of counter-ions and contaminating ions, in the case of lecirelin determination of anions of TFA, TFMSA and acetic acid (AcOH).

Until recently, ion chromatography (IC) using anion-exchange columns and conductivity detectors has been the most widely used method for analysis of low-molecular-mass ionic admixtures (salts) in peptide preparations [19]. Besides IC, also RP-HPLC with silica-based amine stationary phase with conductivity detector (without an ion suppression column) has been used for the separation and quantification of methanesulfonic acid in samples of pharmaceutical intermediates and drug substances [20].

In the recent years, CZE with indirect UV-absorption detection has become an efficient and a reliable method for the determination of anions that have insufficient UV absorption. Various UV-absorbing co-ions of the background electrolyte are components added to the electrophoretic buffer to provide the background UV signal, e.g. 2,6-naphthalendicarboxylic acid used in the analysis of TFA in lyophilized natural products [21], or 4-hydroxybenzoic acid used for the determination of acetate counter-ion in semisynthetic basic lipopeptides (pneumocandins) [22]. It is also worth to mention the validated method for the determination of acetates and trifluoroacetates in synthetic peptide samples using sodium phthalate as the UV-absorbing component of BGE and cetyltrimethylammonium bromide (CTAB) as the electroosmotic flow (EOF) modifier [12,23]. Besides indirect UV-absorption detection, CZE with conductivity detection for the determination of acetate counter-ion in pharmaceutical drug substances was developed and validated [24]. BGE for the determination of acetates contained an amphoteric buffer MES and tetradecyltrimethylammonium hydroxide (TTAOH) modifier to reverse the EOF in the capillary.

The application of CITP in peptide analysis is double useful. Besides the determination of peptide purity degree, it is advantageously used for the determination of anions or cations (counter ions and/or contaminating ions) in peptides. In the pioneering work of van Nispen et al. [25] anions such as formate, citrate, methanesulfonate, trifluoroacetate, acetate, *p*-toluenesulfonate were determined. Special attention was devoted to the determination of acetic acid in peptides, e.g. in N-terminal 24-peptide of adrenocorticotropic hormone, ACTH-(1–24) [26] and a simultaneous determination of AcOH and TFA [18] by anionic CITP with leading electrolyte (LE) 10 mM HCl/His, pH 5.6, and terminating electrolyte (TE) 10 mM MES/Tris, pH 5.6.

Similarly as anionic admixtures, cationic ones were also frequently present in peptide preparations after their synthesis and purification, such as, e.g. cations of the following salts, tetramethylammonium (TMA) phosphate, trimethylamine (TEA) phosphate or formate and ammonium acetate, which were added to the mobile phase during preparative HPLC peptide separations. Concentrations of contaminating cations in peptide preparations during and after purification procedures were checked, e.g. TMA^+ in the analogue of vasopressin, AVP-(1–8) [26], TEA^+ in ACTH-(1–24) [18] using cationic CITP electrolyte system, LE 10 mM KOH/AcOH, pH 4.5, and TE 10 mM β-alanine (BALA)/AcOH to pH 4.5. Thus, CITP was shown to be a valuable technique for the determination of different anionic and cationic admixtures used in the synthesis and purification of peptides. It is a rapid, qualitative and quantitative method for the determination of several anions or cations in a single run.

The aim of this paper was to evaluate peptide purity degree in lecirelin preparations by CZE and cationic CITP and to determine concentration of peptide counter-ions – contaminat-

ing anions of TFMSA, TFA and AcOH in lecirelin batches by anionic CITP.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade. Phosphoric, hydrochloric and acetic acids, and potassium hydroxide were from Lachema (Brno, Czech Republic). Tris (tris(hydroxymethyl)aminomethane) and 2-(*N*-morpholino)-ethanesulfonic acid (MES) were from Serva (Heidelberg, FRG). D,L-Histidine, TFMSA and TFA were obtained from Sigma (St. Louis, MO, USA), and β-alanine (BALA) from Calbiochem (Luzerne, Switzerland). Buffer solutions were prepared from the deionized and redistilled water and filtered through 0.45 μm membrane filter (Millipore, Bedford, USA) prior to the use in CZE and CITP. Different batches of lecirelin were provided by PolyPeptide Laboratories (Prague, CR) and the sample of Dagin was provided by T. Barth (IOCB).

2.2. Capillary zone electrophoresis

CZE experiments were performed in home-made apparatus equipped with UV-absorption photometric detector monitoring absorbance at 206 nm. Fused silica capillary (I.D. 50 μm, O.D. 200 μm, total length 300 mm, effective length 190 mm) was supplied by the Institute of Glass and Ceramics Materials, Academy of Sciences of the Czech Republic (Prague, Czech Republic). The background electrolyte composed of 100 mM H_3PO_4, 50 mM Tris, pH 2.25 was used. Sample solution was applied hydrodynamically (pressure 500 Pa for 5–10 s) and the applied separation voltage (constant) was 10.0 kV (current 50–55 μA). Separations were performed at an ambient temperature of 22–24 °C. Chromatography Station Clarity (DataApex, Prague, Czech Republic) was used for data acquisition and handling.

2.3. Capillary isotachophoresis

CITP experiments were performed in the Electrophoretic Analyser EA 101 (Villa Labeco, Spišská Nová Ves, Slovakia) equipped with column coupling system consisting of two fluorinated ethylene–propylene copolymer (FEP) capillaries. The first, preseparation capillary (160 mm × 0.8 mm I.D.), is connected with the analytical capillary (180 mm × 0.3 mm I.D.) via the bifurcation block, which enables the determination of the sample macrocomponents in the preseparation capillary and the determination of microcomponents in the analytical capillary. Contactless conductivity detectors are placed on both columns 40 mm from the outlet ends, and UV-absorption detector (set to the wavelength 254 nm or 220 nm) is situated 30 mm from the outlet end of the analytical column. Sample volumes 2–5 μl were applied by microsyringe through the septum placed above the injection valve. The driving constant currents were 250 and 50 μA in the preseparation and analytical columns, respectively. Separations were performed at an ambient temperature of 22–24 °C. The composition of the electrolyte systems used is given in Table 1. Data acquisition and handling were performed using software ITP Pro32, version 1.0.5 (J&M Analytische Mess- und Regeltechnik GmbH, Aalen, Germany).

3. Results and discussion

3.1. Determination of purity degree by CZE

Lecirelin is nonapeptide with noncharged C-terminal ethylamide group of proline and pyroglutamic residue on N-terminus. There are three ionogenic groups, imidazol of histidine ($pK_a \sim 6.0$), guanidium group of arginine ($pK_a \sim 11.3$) and hydroxy group of tyrosine ($pK_a \sim 10.4$). The relative molecular mass of lecirelin is 1209.4. The electrophoretic mobility of peptides is directly proportional to their effective charge and indirectly to their size (relative molecular mass). Therefore, dependence of effective charge and specific charge (effective charge divided by relative molecular mass) on pH is the important parameter for the selection of suitable experimental conditions. The course of these dependences for lecirelin, calculated by using the program Nabampho [14] is presented in Fig. 1. From these graphs the suitable experimental conditions, particularly the pH and composition of the BGE, can be derived. From the pH dependencies of effective and specific charges of lecirelin, it follows that this peptide is strongly basic, with rather high isoelectric point (p$I \sim 10.8$), which predetermines it to be analyzed as cation in a rather broad pH range of the BGE. Anionic CZE analysis would be possible only in strongly alkaline BGEs (pH > 11), which are unsuitable BGEs because of their high conductivity, resulting in high temperature increase inside the capillary due to Joule heating.

Lecirelin batch samples were analyzed as cations in acidic Tris-phosphate BGE. The examples of CZE analyses of crude and HPLC purified lecirelin are shown in Fig. 2. Peptide purity was quantified by three ways: (i) relative peak height, $P_h(i)$, defined by Eq. (1), (ii) relative peak area, $P_A(i)$, Eq. (2), and (iii) relative corrected peak area, $P_{CA}(i)$, Eq. (3), of the UV-positive

Table 1
Composition of CITP electrolyte systems

CITP mode	Leading electrolyte (LE)			Terminating electrolyte (TE)		
	Leading ion	Counterion	pH	Terminating ion	Counterion	pH
Anionic	10 mM Cl^-	20 mM His^+	6.0	10 mM MES^-	H^+	4.0
Cationic	10 mM K^+	25 mM AcO^-	4.5	10 mM $BALA^+$	AcO^-	4.4

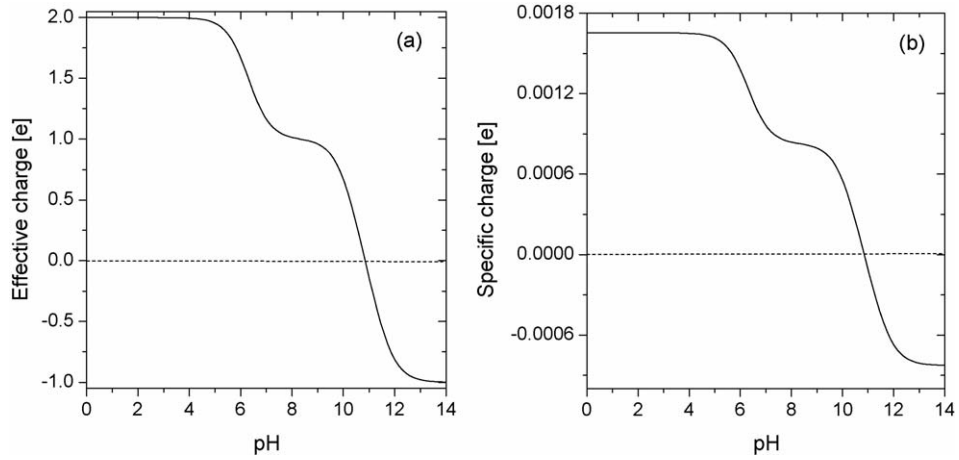

Fig. 1. The pH dependence of effective charge (a) and specific charge (b) of lecirelin (specific charge = effective charge divided by relative molecular mass).

peaks for the *i*th component of the peptide preparation:

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

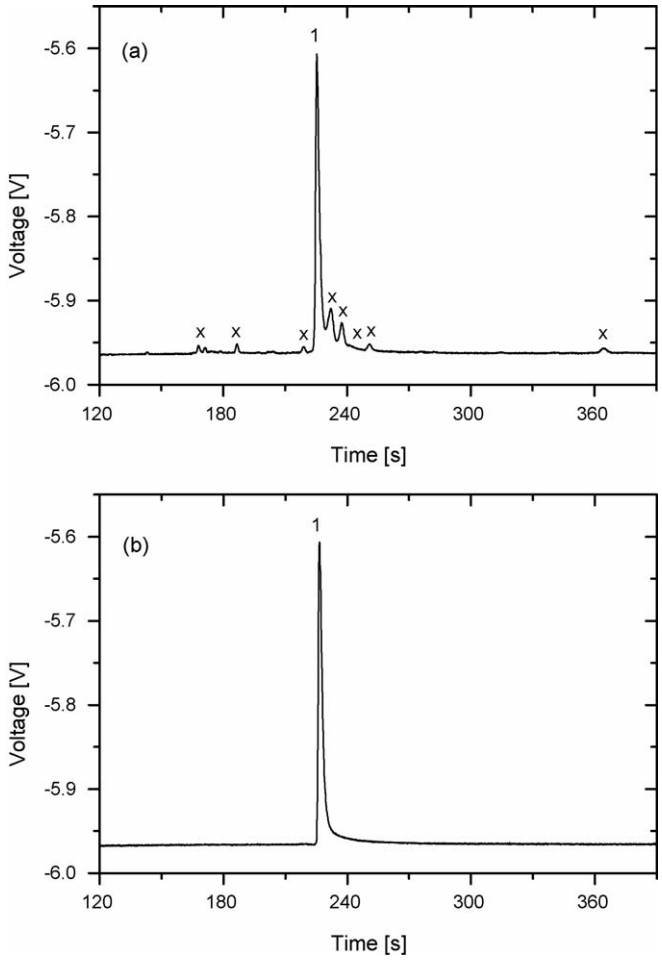

Fig. 2. CZE analyses of crude (a) and HPLC purified (b) lecirelin. 1, lecirelin; *x*, nonidentified admixtures; voltage, signal of UV absorption detector at 206 nm; for the other experimental conditions see the text (Section 2.2).

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

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

where $h(i)$ is the height, $A(i)$ the area, $A_{\mathrm{c}}(i)$ is corrected area of the *i*th peak, corrected peak area is the 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 peak and n is the number of sample components. The values of purity degrees were determined as averages of values obtained in two analyses, which differed less than 1–2%. The values of differently expressed purity degrees of analyzed batches of lecirelin are presented in Table 2. Analyses of purified batches of lecirelin indicate high degree of peptide purity. Analyses of crude batches show significantly higher degree of purity based on peak heights in comparison with those based on peak area. It follows from the fact, that there is relatively small height and broad width of the peaks of many minor admixtures of lecirelin.

Dagin – spawning agent in aquaculture, containing lecirelin in 0.014 mg/kg and metoclopramide in 20 mg/kg of body mass concentrations, respectively, was analyzed in acidic Tris-phosphate BGE as well. Lecirelin was successfully separated

Table 2
Values of purity degree of lecirelin preparations determined by CZE and CITP

Lecirelin batch no.	CZE			CITP	
	P_{h} (%)	P_{A} (%)	P_{CA} (%)	P_{LC} (%)	P_{LUV} (%)
Crude					
1	69.3	60.1	60.6	63.9	71.4
2	71.4	64.4	66.6	72.4	81.4
3	73.9	67.2	69.1	79.8	80.9
HPLC purified					
1	99.9	99.9	99.9	96.4	97.5
2	99.9	99.9	99.9	98.8	97.6
3	99.9	99.9	99.9	99.9	97.6
4	99.9	99.9	99.9	99.9	98.6

P_{h}, P_{A}, P_{CA}, P_{LC}, P_{LUV} are the different types of purity degrees defined by Eqs. (1)–(5).

from the excess of metoclopramide (4-amino-5-chloro-*N*-[2-(diethylamino)ethyl]-2-methoxybenzamide). Migration times of metoclopramide ($t = 177.4$ s) and lecirelin ($t = 224.7$ s) show that electrophoretic mobilities of these analytes are different enough for complete separation (see Fig. 3), even at three orders higher concentration of metoclopramide. This is a suitable example of the determination of peptide in the presence of few orders higher excess of low-molecular-mass compound.

3.2. Determination of purity degree by CITP

Lecirelin batch samples were analyzed in cationic ITP mode. The examples of CITP analyses of crude and purified lecirelin are shown in Fig. 4. Peptide purity was quantified by relative isotachophoretic zone length, $P_{LC}(i)$, defined by Eq. (4) (conductivity detector) and $P_{LUV}(i)$, defined by Eq. (5) (UV-detector) of the ith component of the peptide preparation.

$$P_{LC}(i) = \frac{L_C(i)}{\sum L_C(i)}, \quad i = 1, \ldots, n \tag{4}$$

$$P_{LUV}(i) = \frac{L_{UV}(i)}{\sum L_{UV}(i)}, \quad i = 1, \ldots, n \tag{5}$$

where $L_C(i)$ and $L_{UV}(i)$ are the lengths of ith zone in the conductivity or UV-absorption detector, respectively, and n is the total number of isotachophoretic sample zones between LE and TE. The values of purity degrees were determined as averages of values obtained in two analyses, which differed less than 2%. The values of purity degrees $P_{LC}(i)$ and $P_{LUV}(i)$ of analyzed batches of lecirelin are presented in Table 2. Analyses of purified batches of lecirelin indicate a little bit higher degrees of peptide purity calculated from the results of conductivity detec-

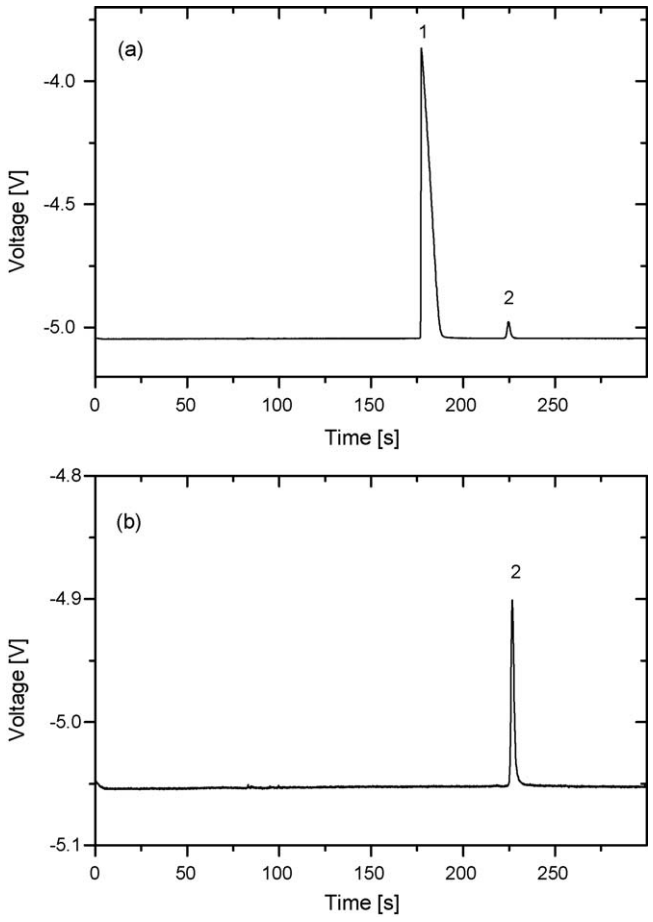

Fig. 3. (a) CZE analysis of Dagin 13, spawning agent, containing lecirelin in the presence of three orders higher concentration of metoclopramide and (b) CZE analysis of lecirelin standard. 1, metoclopramide; 2, lecirelin; the experimental conditions as in Fig. 2.

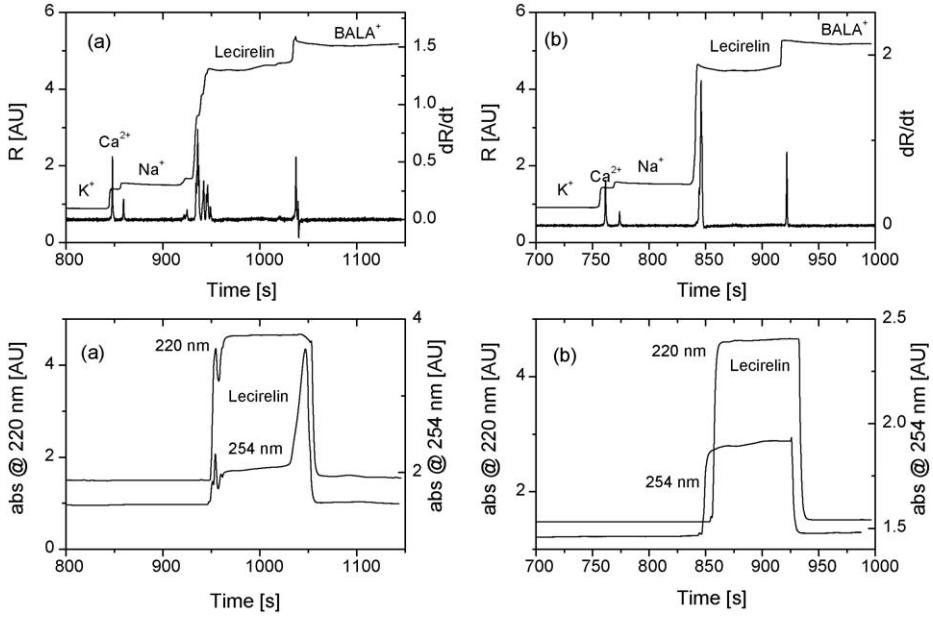

Fig. 4. Conductivity detector records (integral plot and first derivative) (top), and UV-absorption detector records (at 220 and 254 nm) (bottom) of cationic CITP analyses of lecirelin preparations. (a) Crude batch no. 1 and (b) HPLC purified batch no. 3. R, resistance in arbitrary units; dR/dt, first derivative of the conductivity detector signal; abs @ 220 nm, absorbance at 220 nm; abs @ 254 nm, absorbance at 254 nm. Sample volume 3 μl; the other experimental conditions are given in the text (Section 2.3).

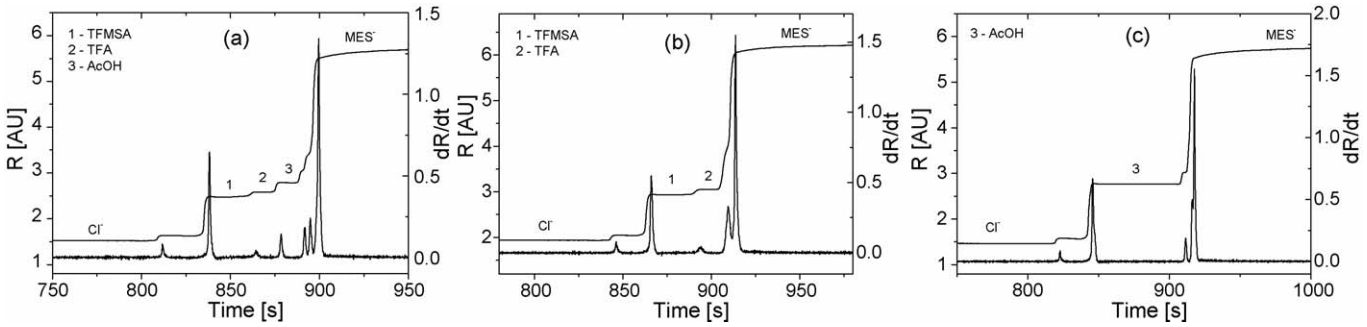

Fig. 5. Conductivity detector records (integral plot and first derivative) of anionic CITP analyses of crude (a and b, batches nos.1 and 3) and purified (c, batch no. 3) samples of lecirelin. The experimental conditions as in Fig. 4.

tor than those of UV detector. It means that some UV-absorbing minor impurities are present, which are not resolved as individual zones by conductivity detector. Analyses of crude batches 1 and 2 show rather small degrees of purity calculated from the results of conductivity detector in comparison with those of UV detector because of the presence of some non-UV absorbing admixtures with higher effective mobilities than that of lecirelin. UV detector results obtained from two different wavelengths show that there is an important admixture in crude samples with higher absorption coefficient at $\lambda = 254$ nm and smaller effective mobility in comparison with those of lecirelin. Moreover, lecirelin possesses higher absorption coefficient at $\lambda = 220$ nm than at $\lambda = 254$ nm.

Table 3
Relative step heights (RSH) and calibration curves equations of the CITP determination of anions of contaminating acids in lecirelin batches in anionic electrolyte system given in Table 1 (LE–10 mM HCl, 20 mM His, pH 6.0; TE–10 mM MES, pH 4.0)

Acid	RSH	Calibration curve equation	R^2_{xy}
TFMSA	0.235 ± 0.004	$y = 2.70x + 1.64$	0.995
TFA	0.262 ± 0.006	$y = 2.90x + 1.23$	0.998
AcOH	0.312 ± 0.006	$y = 3.02x + 0.34$	0.996

RSH, relative step height; y, zone length (s); x, amount of substance (nmol); R_{xy}, correlation coefficient.

Table 4
Concentration of contaminating acids in lecirelin batches determined by CITP in anionic mode

Lecirelin batch no.	Mass percentage (%)[a]		
	TFMSA	TFA	AcOH
Crude			
1	5.5	2.2	1.2
2	7.9	2.7	0.6
3	8.8	2.8	0
HPLC purified			
2	0	0.3	8.7
3	0	0	11.5
4	0.6	0	9

[a] Averaged values from two ITP analyses, the values of which differed less than 2–3%.

3.3. Determination of counter-ion content by CITP

Anionic low molecular mass admixtures, the counter-ions of the basic peptide lecirelin, were determined by anionic CITP in several lecirelin batch samples. The examples of different analyses of crude and purified batches of lecirelin are shown in Fig. 5. Quantitative determination of TFMSA, TFA and AcOH was carried out by the calibration curve method from the lengths of appropriate zones. The equations of the calibration curves, correlation coefficients and the relative step heights of the above anions are presented in Table 3. Resulting concentrations of contaminating acids (in mass percentages) are compiled in Table 4.

4. Conclusions

1. CZE and CITP proved to be high-efficient and high-sensitive methods for qualitative and quantitative analysis of different preparations of lecirelin (synthetic peptide veterinary drug) in the nanomole (CITP) to femtomole (CZE) level.
2. CZE was found to be more suitable for peptide purity determination of lecirelin preparations than CITP due to its higher separation power.
3. CITP with contactless conductivity detection was found to be more suitable for the determination of low-molecular-mass anionic contaminating admixtures (acetates, trifluoroacetates, trifluoromethanesulfonates) in lecirelin preparations than CZE with fixed-wavelength (206 nm) UV-absorption detection due to the universal character of the conductivity detection capable to detect zones of the above non-UV-absorbing compounds.

Acknowledgements

The work was supported by the Grant Agency of the Czech Republic, grants no. 203/05/2539, 203/03/0716, 203/04/0098, and by the Research Project Z40550506 of the Academy of Sciences of the Czech Republic. Mrs. V. Lišková is thanked for her skilful technical assistance.

References

[1] M.W. Monahan, M.S. Amoss, H. Anderson, W. Vale, Biochemistry 12 (1973) 4616.

[2] R.H. Rippel, E.S. Johnson, W.F. White, M. Fujino, I. Yamazaki, R. Nakayama, Endocrinology 93 (1973) 1449.
[3] M. Flegel, J. Pospíšek, J. Picha, in: K. Bláha, P. Maloň (Eds.), Peptides 1982, Walter de Gruyter, Berlin, 1983, p. 551.
[4] R. Doležal, R. Novotný, S. Čech, J. Zajíc, V. Havlíček, Acta Vet. (Brno) 69 (2000) 289.
[5] P.S. Baruselli, R. Amaral, F.B. Barufi, R. Valentim, M.-O. Marques, Braz. J. Vet. Res. Anim. Sci. 38 (2001) 142.
[6] R.E. Peter, H.R. Lin, G. Van der Kraak, Aquaculture 74 (1988) 1.
[7] E. Brzuska, Aquac. Res. 35 (2004) 1318.
[8] E. Brzuska, Aquac. Res. 36 (2005) 1015.
[9] T. Szabo, Aquac. Res. 34 (2003) 479.
[10] E. Brzuska, J. Kouřil, J. Adámek, Z. Stupka, V. Bekh, Czech J. Anim. Sci. 49 (2004) 297.
[11] V. Kašička, Electrophoresis 24 (2003) 4013.
[12] S. Ridge, K. Hettiarachchi, J. Chromatogr. A 817 (1998) 215.
[13] V. Šolínová, V. Kašička, T. Barth, L. Hauzerová, S. Fanali, J. Chromatogr. A 1081 (2005) 9.
[14] V. Kašička, Z. Prusík, J. Chromatogr. 470 (1989) 209.
[15] A. Kopwillem, F. Chillemi, A.B. Bosisio-Righetti, P.G. Righetti, in: H. Peeters (Ed.), Protides of Biological Fluids, Pergamon Press, Oxford, New York, 1974, p. 657.
[16] R. Jannasch, Pharmazie 38 (1983) 379.
[17] V. Kašička, Z. Prusík, in: H. Parvez, P. Caudy, S. Parvez, P. Roland-Gosselin (Eds.), Capillary Electrophoresis in Biotechnology and Environmental Analysis, VSP, Utrecht, 1997, p. 173.
[18] P.S. Janssen, J.W. van Nispen, M.J. van Zeeland, P.A. Melgers, J. Chromatogr. 470 (1989) 171.
[19] E. Kaiser, J. Rohrer, J. Chromatogr. A 1039 (2004) 113.
[20] B.S. Lord, R.W. Stringham, Anal. Chem. 68 (1996) 1067.
[21] M.A. Strege, W.G. Mascher, J. Chromatogr. B 697 (1997) 255.
[22] L. Zhou, A. Dovletoglou, J. Chromatogr. A 763 (1997) 279.
[23] K. Hettiarachchi, S. Ridge, J. Chromatogr. A 817 (1998) 153.
[24] R.C. Wiliams, R. Boucher, J. Brown, J.R. Scull, J. Walker, D. Paolini, J. Pharm. Biomed. Anal. 16 (1997) 469.
[25] J.W. van Nispen, P.S. Janssen, B.C. Goverde, J.C. Scherders, F. van Dinther, J.A. Hannink, Int. J. Pept. Protein Res. 17 (1981) 256.
[26] P.S.L. Janssen, J.W. van Nispen, J. Chromatogr. 287 (1984) 166.