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Separation of biologically active peptides by capillary electrophoresis and high-performance liquid chromatography

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Abstract

HPLC and CE have been applied to the separation of some newly synthesized substances, including nonapeptides from the intrachinary region of insulin, insulin-like growth factors I and II (IGF I and II) and some penta- and hexapeptides. All the peptides are satisfactorily separated using a reversed-phase HPLC system with a C_{18} stationary phase and mobile phases of 20–40% acetonitrile (v/v) and 0.2% trifluoroacetic acid in water (v/v). The best CE separation of IGF I and II has been achieved in a 30 m*M* phosphate buffer (pH 4–5), whereas 150 m*M* phosphoric acid (pH 1.8) is optimal for the insulin nonapeptides. The latter electrolyte is also suitable for the CE separation of the hexapeptides, as is a micellar system containing 20 m*M* borate–50 m*M* sodium dodecyl sulfate (pH 9.0). Complete CE resolution of the D- and L-forms is possible in a 50 m*M* phosphate buffer (pH 2.5) containing 10 m*M* β-cyclodextrin. UV spectrophotometric detection was used throughout, at wavelengths from 190 to 215 nm. The CE procedures are, in general, preferable to HPLC separations, as they exhibit better separation efficiencies, are faster and consume smaller amounts of analytes and reagents.

Keywords: Peptides

1. Introduction

In view of the great importance of naturally occurring peptides in the regulation of processes in living organisms, many of these substances and their analogues have been synthesized, to facilitate the study of life and to affect the functioning of organisms, e.g., in biology, medicine, or agriculture. For this reason it is necessary to check the identity and purity of such preparations and to investigate their fate, as well as the products of their metabolism. This is achievable only by use of high-performance separation techniques.

This paper deals with some synthetic penta-, hexa- and nonapeptides, namely, analogues of enkephalin (dalargin) and of the nonapeptides from the intrachinary region of human insulin and the insulin-like growth factors, IGF I and II. The most important methods for their separation are high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Thus far, hexapeptides of this type have been separated by reversed-phase [1–3], ion-exchange [4] and size-exclusion [2] HPLC and, more recently, by CE (e.g., Refs. [5–7]) and micellar electrokinetic chromatography (MEKC) [7]. For nonapeptides, ion-exchange [8–10], reversed-phase [8–11] and size-exclusion [10] HPLC, and CE [8,9,11–13] have been employed. We have applied

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the experimental design described in the literature cited above to optimize the conditions for separations of newly synthesized substances and tested the usefulness of reversed-phase HPLC and CE in various systems.

2. Experimental

2.1. Apparatus

The HPLC measurements were carried out using an integrated system of a Crystal Model 200 instrument (pump+autosampler or manual injection system) with a diode-array detector Model 250 and a Dell data station (ATI Unicam, Cambridge, UK). The columns used were the following: (1) Separon RP-18 SGX column (150×3.3 mm I.D., 7 μm particle size; Tessek, Prague, Czech Republic); (2) Vydac RP-18 column (250×4.6 mm I.D., 5 μm particle size; Vydac, Hesperia, CA, USA); (3) Separon HEMA S-1000 RP-18, poly(hydroxy ethyl methacrylate onto which the C_{18} chains had been covalently bound) (150×3.3 mm I.D., 10 μm particle size; Tessek); (4) LiChrosorb RP-18 (150×3.0 mm I.D., 5 μm particle size; Merck, Darmstadt, Germany).

Sample volumes of 10 and 25 μl were injected Onto the HPLC system.

The CE separations were performed with a Crystal Model 310 system, with a Model 4225 UV detector and a Dell 433/M data station (ATI Unicam). The fused-silica capillary, 75 mm I.D., was 65 cm long and the distance to the detector was 50 cm. Pneumatic sampling was employed for 3–6 s and at 100–300 Pa, depending on the sample concentration.

UV spectrophotometric detection was carried out in the range between 190 and 215 nm, depending on the analyte spectra. All the measurements were performed at laboratory temperature. The HPLC mobile phases were degassed by passage of helium prior to chromatography.

2.2. Chemicals

The substances studied are listed in Table 1. They were synthesized on a solid-phase using usual condensation and deprotection reactions [6,14,15], purified by HPLC or free-flow zone electrophoresis (FFZE) [6,14,16] and lyophilized. The preparations thus obtained were dissolved in deionized water immediately prior to separation procedures. All other chemicals were of analytical grade, obtained from various manufacturers, and were not further purified. Deionized water was used throughout.

3. Results and discussion

Taking into account the character of the studied substances, two methods for their separation have been selected: the molecules are not large enough in size for application of size-exclusion chromatography. Futhermore, they do not contain sufficient ionizable groups for ion-exchange HPLC. Therefore, flexible reversed-phase HPLC seems to be the best alternative. Capillary electrophoresis is rapidly gaining importance in this field and thus is an obvious alternative to HPLC.

3.1. Nonapeptides

HPLC separation was tested using a C_{18} stationary phase. An aqueous acetonitrile (ACN) mobile phase containing 0.2% trifluoroacetic acid (TFA) was selected on the basis of literature data [2,8]. As expected, retention times increased with decreasing content of the organic modifier and 20% ACN allowed separation of the nonapeptides within an adequate time (see Fig. 1). However, the separation efficiency was not as satisfactory as required. Moreover, nonapeptide IX was not eluted at all.

In the CE separation, the effects of the buffer concentration, pH and the applied voltage were first examined. From the point of view of the Joule heat generated, the buffer concentration should be as low as possible, and a 30 m*M* phosphate buffer proved to be the optimum choice. The voltage applied should be as high as possible, but should be within a linear voltage–current dependence; a voltage of 20 kV was selected for the buffers used. The magnitude of the electroosmotic flow (EOF) was determined using thiourea as the marker; the results as a function of pH are given in Table 2. The reproducibility of the migration times was rather poor, presumably due to adsorption of the test substances on the capillary

Table 1
Studied peptides

Nonapeptides	
From intrachinary region A_{6-14} of insulin	
I	Cys-Cys(Acm)-Thr-Ser-Ile-Cys-Ser-Leu-TyrNH$_2$
II	Cys-Cys(Acm)-Thr-Ser-Ile-Cys-Ser-Leu-Tyr
III	Cys-Ala-Thr-Ser-Ile-Cys-Ser-Leu-TyrNH$_2$
IV	Cys-Ala-Thr-Ser-Ile-Cys-Ser-Leu-Tyr
From intrachinary region A_{6-14} of IGF I	
V	Cys-Cys(Acm)-Phe-Arg-Ser-Cys-Asp-Leu-Arg
VI	Cys-Ala-Phe-Arg-Ser-Cys-Asp-Leu-Arg
VII	Cys-Cys(Acm)-Phe-Arg-Ser-Cys-Asp-Leu-ArgNH$_2$
VIII	Cys-Ala-Phe-Arg-Ser-Cys-Asp-Leu-ArgNH$_2$
From intrachinary region A_{6-14} of IGF II	
IX	Cys-Cys (Acm)-Phe-Arg-Ser-Cys-Asp-Leu-Ala
X	Cys-Ala-Phe-Arg-Ser-Cys-Asp-Leu-Ala
XI	Cys-Cys(Acm)-Phe-Arg-Ser-Cys-Asp-Leu-AlaNH$_2$
XII	Cys-Ala-Phe-Arg-Ser-Cys-Asp-Leu-AlaNH$_2$
Penta- and hexapeptides	
Dalargin analogues	
XIII	Dalargin Tyr-D-Ala-Gly-Phe-Leu-Arg
XIV	Dalargin ethylamide Tyr-D-Ala-Gly-Phe-Leu-ArgNHEt
XV	[Methionin]^{5}dalargin Tyr-D-Ala-Gly-Phe-Met-Arg
XVI	[D-Neopentylglycine]^{5}dalargin Tyr-D-Ala-Gly-Phe-D-Npl-Arg
XVII	[L-Neopentylglycine]^{5}dalargin Tyr-D-Ala-Gly-Phe-L-Npl-Arg
XVIII	[D-di*tert.*Leucine]^{2,5}dalargin Tyr-Tle-Gly-Phe-Tle-Arg
XIX	[D-*tert.*Leucine]^{5}dalargin Tyr-D-Ala-Gly-Phe-D,L-Tle-Arg
XX	[N-CH$_3$-L-Phe]^{4}dalargin Tyr-D-Ala-Gly-[N-CH$_3$-L-Phe)-Leu-Arg
XXI	(N-CH$_3$-D-Phe]^{4}dalargin Tyr-D-Ala-Gly-[N-CH$_3$-D-Phe]-Leu-Arg
XXII	[L-Ala]^{2}dynorphin Tyr-Ala-Gly-Phe-Leu-Arg
Enkephalin analogues	
XXIII	Methionine-enkephalin Tyr-Gly-Gly-Phe-Met
XXIV	Enkephalin PheOAc-Tyr-Gly-Gly-Phe-LeuOMet

walls; this problem was overcome by washing the capillary for several minutes with 0.1 *M* NaOH after each series of four to five measurements.

On the basis of the above results, various electrolyte systems were tested for the separation of nonapeptides:

(1) Nonapeptides V and VI, containing arginine with a free carboxylic group at the C-end, are best separated in 30 m*M* KH_2PO_4 (pH 4); however, the electropherograms are very complex and suggest that the synthesis did not yield well-defined products.

(2) Nonapeptides VII and VIII, containing arginine

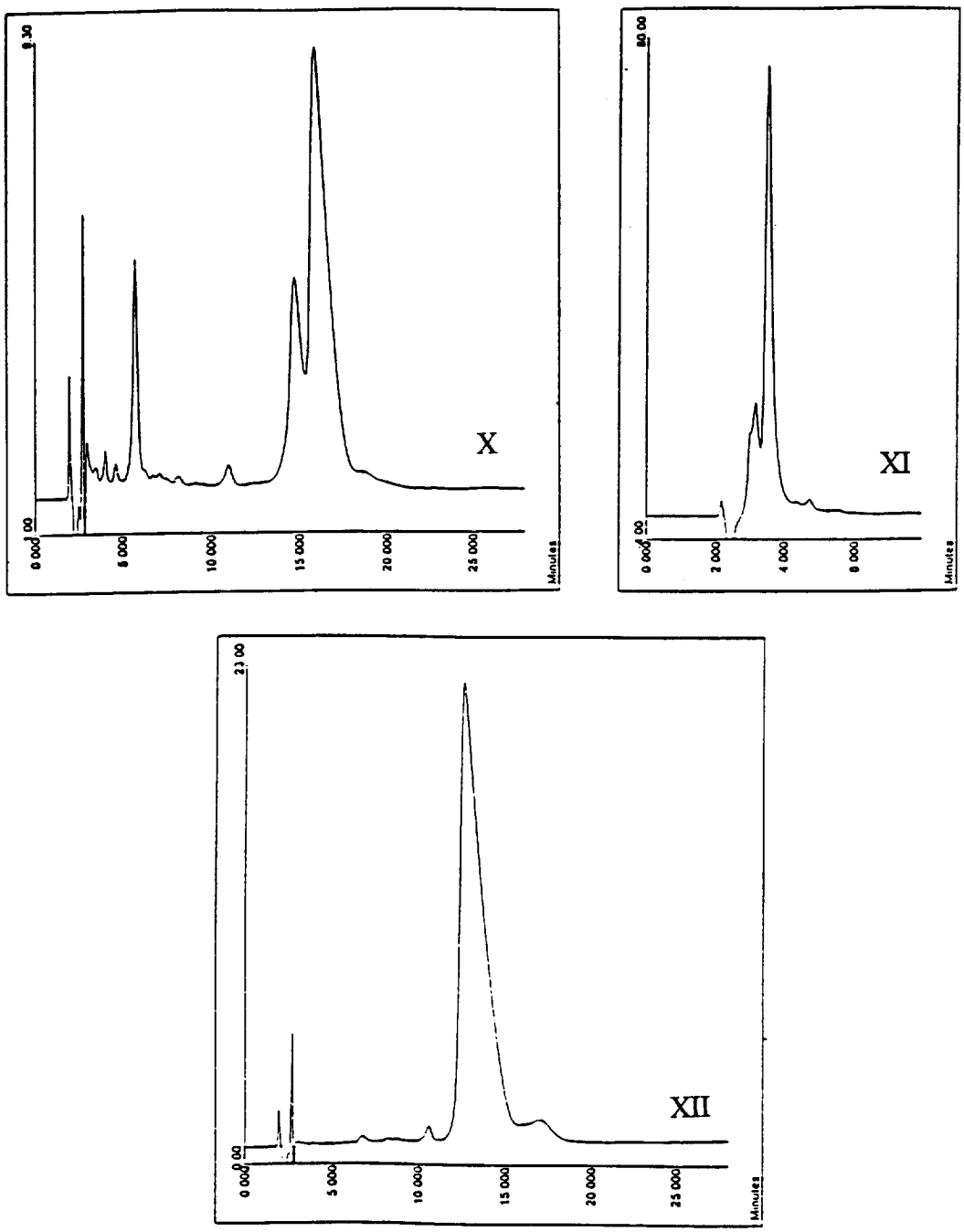

Fig. 1. HPLC analysis of nonapeptides X, XI and XII. Stationary phase, Separon RP-18 SGX; mobile phase, 20% ACN+0.2% TFA (pH 2.2).

Table 2
Dependence of the EOF on the pH

pH	t_o (min)	R.S.D. (%)	V_{eo} (cm/s)	m_{eo} ($cm^2/V \cdot s$)
4.03	36.3	4.71	$1.84 \cdot 10^{-2}$	$5.05 \cdot 10^{-5}$
5.00	34.5	0.72	$1.93 \cdot 10^{-2}$	$5.3 \cdot 10^{-5}$
6.00	7.6	0.12	$8.77 \cdot 10^{-2}$	$2.4 \cdot 10^{-4}$
9.36	5.05	0.03	$1.31 \cdot 10^{-1}$	$3.6 \cdot 10^{-4}$

Electrolyte, 30 m*M* phosphate buffer (pH 4–6) or 20 m*M* borate–50 m*M* SDS (pH 9.34); L_D=40 cm; I.D.=75 μm; pneumatic injection, 100 Pa for 6 s.

with an amide group, are best separated using the same system as above, but at pH 5.0. The peaks are symmetric and the separation efficiency is high (the number of theoretical plates exceeds 10^5; see Fig. 2). This electrolyte is also suitable for nonapeptides IX–XII (Fig. 3).

The nonapeptides containing an amino acid with a free amide group at the C-terminus migrate faster than those with a free carboxyl group, as their positive charge is higher. The compounds containing alanine in position 2 migrate faster than the analogues containing cysteine with a protective group.

The buffer pH affects the retention order as well as the separation efficiency. The optimum pH lies between 2.1 and 5.0. At higher pH values the resolution decreases markedly and furthermore, the baseline suffers from a high noise.

For separation of the nonapeptides A6–A14 of the sequence of human insulin, more concentrated electrolyte systems have been found to be convenient, namely 150 m*M* phosphate buffers. At a pH of 4.7, only nonapeptides I and III with a terminal amide were detected, whereas those with free C-termini did not elute. All the peptides were detected in 150 m*M* phosphoric acid (pH 1.8). Again, peptides with a terminal amide group (I and III) migrate faster than those with free carboxyls (II and IV) and the retention times of those with alanine in position 2 are

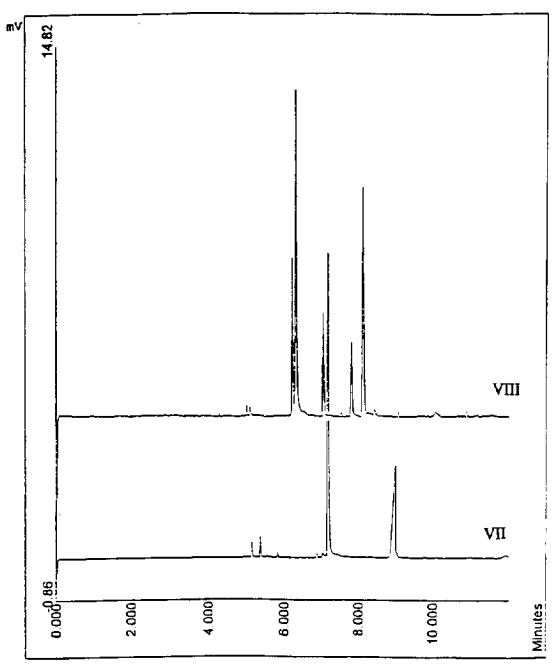

Fig. 2. CE analysis of nonapeptides VII and VIII in 30 m*M* KH_2PO_4 (pH 5.0).

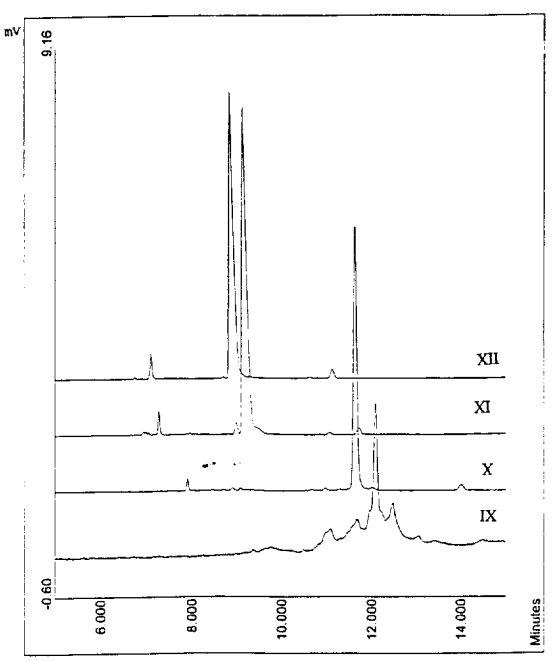

Fig. 3. CE analysis of nonapeptides IX, X, XI and XII in 30 m*M* KH_2PO_4 (pH 5.0).

shorter than for those with protected cysteine (Fig. 4a,b).

3.2. Hexapeptides

The separation efficiency of reversed-phase HPLC improved with increasing concentration of ACN in the aqueous mobile phase containing 0.2% TFA (pH

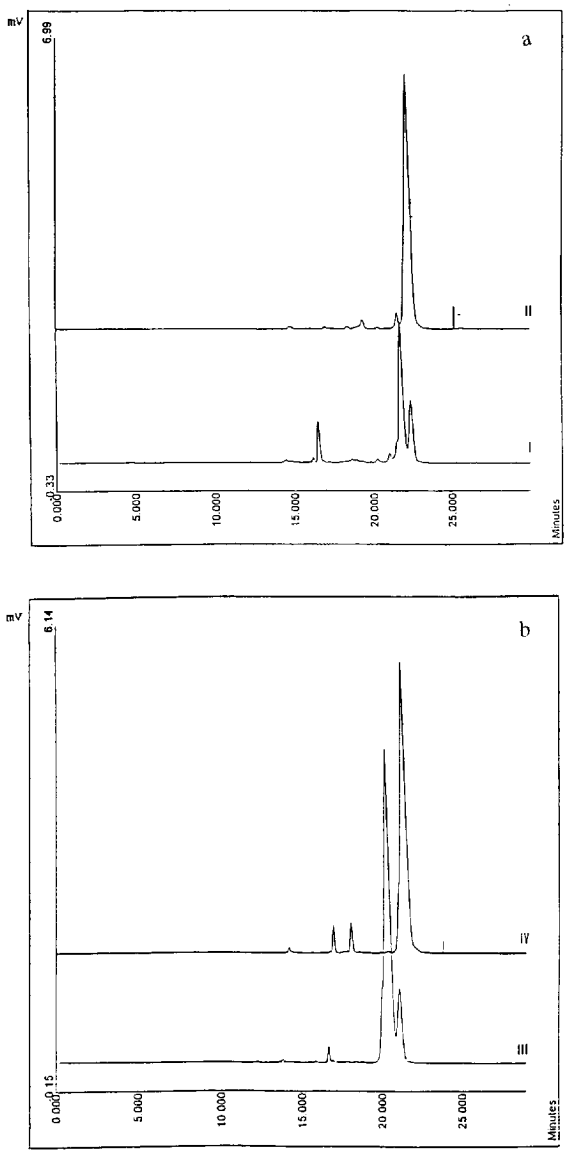

Fig. 4. CE analysis of nonapeptides I and II (a), and III and IV (b) in 150 m*M* phosphoric acid (pH 1.8).

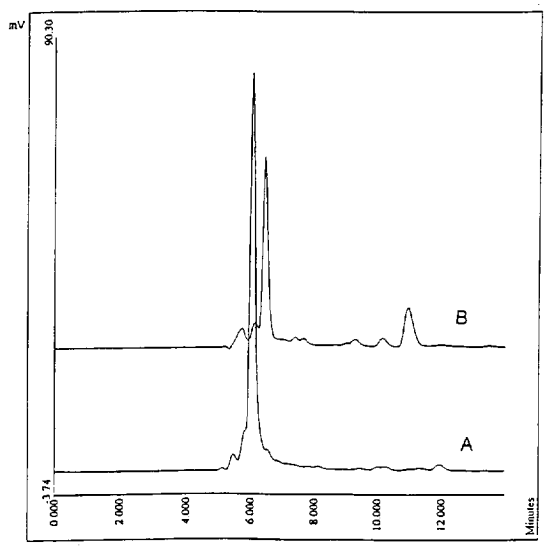

Fig. 5. HPLC separation of [N-CH_3-L-Phe][4] (A) and [N-CH_3-D-Phe][4] (B) dalargins. Stationary phase, Vydac RP-18; mobile phase, 40% ACN+0.2% TFA (pH 2.3).

2.3). However, separation is not as satisfactory as with the CE technique. Nevertheless, chiral separations are possible (Fig. 5).

For CE separation of these substances, phosphate buffers, citric acid and sodium borate with sodium dodecyl sulfate were tested, with the pH varying from 1.8 to 9.0. An optimum separation was achieved in a micellar system of 20 m*M* sodium borate and 50 m*M* sodium dodecyl sulfate at pH 9.0 (Fig. 6). Additionally, chiral separation of the D- and L-forms was possible either in 150 m*M* phosphoric acid, pH 2.0 (a high number of theoretical plates, 2.5×10^5, was calculated) or in a 50 m*M* phosphate buffer (pH 2.5) containing 10 m*M* β-cyclodextrin (Fig. 7).

4. Conclusions

Both reversed-phase HPLC and CE can be used for separations of the substances from the class of short and medium-length peptides, but CE is usually preferable, as the separation efficiency is higher, the

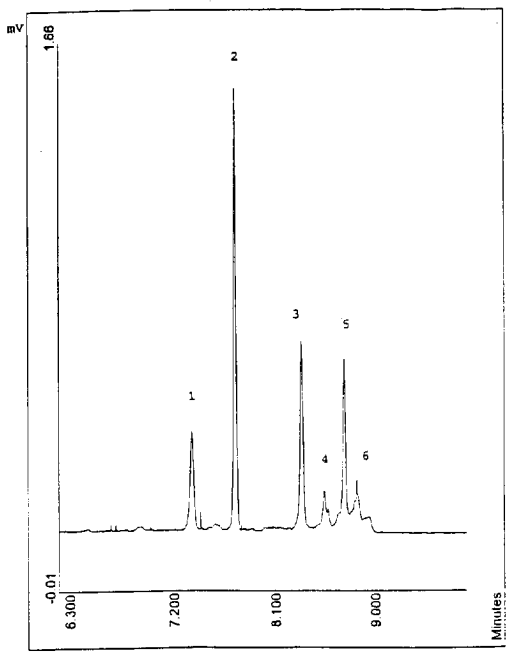

Fig. 6. CE separation of dalargins in 20 m*M* borate–50 m*M* SDS (pH 9.0). Peaks: 1=[Met][5] dalargin; 2=dalargin; 3=[L-Npl][5] dalargin; 4=[Tle][5] dalargin; 5=[D-Tle][2,5] dalargin; 6=dalargin ethylamide.

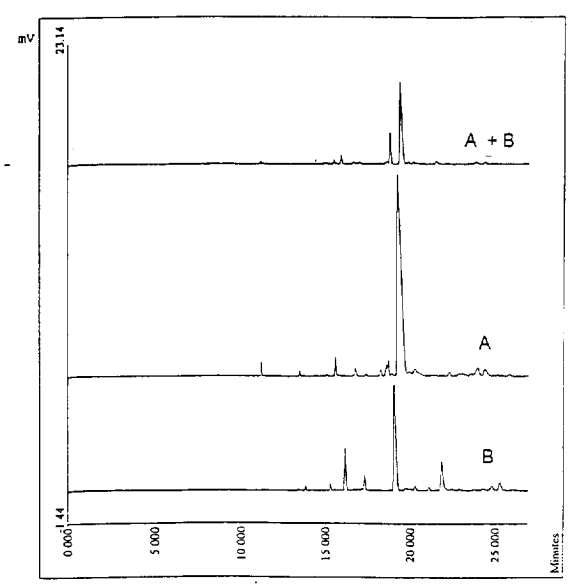

Fig. 7. CE separation of [N-CH_3-L-Phe][4] (A) and [N-CH_3-D-Phe][4] (B) dalargins in 10 m*M* β-cyclodextrin in a 50 m*M* phosphate buffer (pH 2.5).

separation is accomplished within a shorter time and the consumption of either sample or reagents is much smaller. It is likely that the parameters of the HPLC procedure might be somewhat improved by optimizing the mobile phase composition, using other buffer systems and/or employing gradient elution. Unfortunately, these experiments could not be performed because of very limited amounts of the test analytes available (less than 1 mg).

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