ELSEVIER

Journal of Chromatography A 817 (1998) 215–222

JOURNAL OF CHROMATOGRAPHY A

Peptide purity and counter ion determination of bradykinin by high-performance liquid chromatography and capillary electrophoresis

Shane Ridge, Kanthi Hettiarachchi*

Analytical Chemistry Department, Biopharmaceutical Development Division, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA

Abstract

Bradykinin is a nonapeptide hormone of physiological importance. This peptide can be synthesized in yields as high as 83% by solid-phase synthesis. The work presented in this paper describes peptide purity determination of synthetic bradykinin acetate by both reversed-phase HPLC and CE. For purity determination by HPLC, two types of reversed-phase columns were used with gradient elution. The solvent system consisted of trifluoroacetic acid as the ion-pairing reagent and acetonitrile as the organic modifier. An LPA coated capillary and phosphate–phosphoric acid electrolytes of varied pH values were employed to determine purity by CE. The results obtained from these experiments demonstrated the high resolving power of CE in comparison to HPLC for peptide analysis. In CE, resolution of bradykinin and its impurities was greatly improved by varying the electrolyte pH. CE was also used to determine the content of acetic acid in the bradykinin sample. This was performed using a fused-silica capillary and a buffer consisting of sodium phthalate as the UV-absorbing background electrolyte and cetyltrimethylammonium bromide as the electroosmotic flow modifier under an indirect UV detection mode.

Keywords: Peptides; Bradykinin; Hormones

1. Introduction

Discovery of new, biologically active peptides over the past few decades boosted the need for simplified and rapid methods for manufacturing peptides. For the synthesis of larger peptides or small proteins, conventional methods available are not efficient with respect to time and yield. This is because the losses incurred upon isolation and purification of the reaction product during each of the many steps contribute significantly to the low yields of final polypeptide. Addressing these needs, Merrifield in 1962 introduced solid-phase peptide synthesis (SPPS) by synthesizing first a tetrapeptide [1] and then bradykinin, a nonapeptide [2,3]. In SPPS, isolation and purification of each reaction product during building up of the polypeptide chain is precluded by covalently anchoring the growing polypeptide chain to an insoluble solid support such as beads of polystyrene resin. This permits almost quantitative recovery and purification of intermediate products by simply filtering and washing the beads. Subsequently, SPPS was developed into automation [4,5] that indeed caused a revolution in entire peptide field and its influence spread to other areas such as oligonucleotide synthesis. Bradykinin is among several peptides that were synthesized for testing the operation of the instrument designed for automated SPPS [5].

Bradykinin is a peptide hormone that has many pharmacological actions, including induction of acute arterial hypotension, vasodilation, increased

*Corresponding author.

0021-9673/98/$19.00
PII: S0021-9673(98)00313-6

capillary permeability, leucocite migration and accumulation, and pain [6]. Its amino acid sequence is Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg (all residues in the L-form). Conventional methods were available for the synthesis of bradykinin [7–9] prior to the development of SPPS. However, in comparison to these conventional methods, an impressive yield of 83% for the crude product and a yield of 68% for the final product after purification by ion-exchange chromatography have been achieved by SPPS [3].

Peptides are manufactured increasingly for various purposes. Those synthesized for therapeutic uses or preclinical investigations must be rigorously tested for their purity. Synthetic peptides may contain closely related peptide impurities resulting from incomplete reactions or numerous side reactions. Preparative HPLC is a frequently used method to purify peptides, however, co-elution of peptide impurities along with the major peptide may occur during such purification. Obviously, analysis of peptides for purity checks should not be performed using the same HPLC method that was used for peptide purification, because homogeneity results derived from such a system can lead to incorrect and misleading information. Furthermore, peptides exhibit very similar UV spectra comprised of a large end absorption band, and depending upon the presence of certain amino acid residues such as phenylalanine, tyrosine, and tryptophan, a very weak maximum near 275–280 nm. Therefore, if a certain HPLC peak represents two peptides that are co-eluted, peak purity data based on UV spectral information derived from a photodiode array detector may indicate sample homogeneity which is an absolutely incorrect conclusion.

Incorporation of another separation technique such as CE that gives orthogonal information is most rewarding in peptide and protein analysis. As demonstrated by Grossman et al., [10], combination of reversed-phase HPLC and CE have produced unambiguous verification of each of the tryptic digest fractions of human growth hormone, and therefore, the identity of the sample. Klushnichenko et al., [11], Hynek et al., [12], Pacákova et al., [13], and Vinther et al., [14] have further demonstrated that simultaneous application of both HPLC and CE provided very convincing results in peptide analysis.

Basic peptides including bradykinin are commonly isolated in the form of salts such as acetates and trifluoroacetates. The formulations prepared for biological evaluations or therapeutic purposes are based on the peptide content. Therefore, it is of paramount importance that the composition of the peptide, including its counter ion content, be accurately determined.

In this work, the peptide purity of a commercially available sample of bradykinin acetate was determined by HPLC and CE. In addition, the applicability of a validated method for determining counter ions of synthetic peptides [15] was tested by evaluating acetic acid content of bradykinin acetate.

2. Experimental

2.1. Instrumentation

The peptide purity determination was performed by HPLC using an HP 1100 HPLC system consisting of quaternary pump, online vacuum degasser, autoinjector, column thermostat, diode-array detector and HP ChemStation for system control and data collection and analysis (Hewlett–Packard, Wilmington, DE, USA). Two HPLC columns, both from Vydac (Hesperia, CA, USA): (1) Vydac 218TP54, a C_{18} 250×4.6 mm analytical column, 5 μm average particle size and 300 Å average pore size; and (2) Vydac 201HS54, a C_{18} 250×4.6 mm analytical column, 5 μm average particle size and 90 Å average pore size were used in these experiments.

CE was performed by using an HP ^{3D}CE system with a built-in diode-array detector and HP ChemStation for system control and data collection and analysis (Hewlett–Packard). LPA coated capillaries were purchased from Bio-Rad (Hercules, CA, USA) and fused-silica capillaries were purchased from J and W Scientific (Folsom, CA, USA).

2.2. Reagents

2.2.1. HPLC

For HPLC experiments, acetonitrile (ACN), HPLC-grade from Mallinckrodt, (Paris, KY, USA) and trifluoroacetic acid (TFA), sequanal-grade from

Pierce (Rockford, IL, USA) were used. The mobile phases were prepared in Milli-Q water.

2.2.2. CE

For CE peptide purity determination, electrolyte solutions were prepared using monobasic potassium phosphate. Analytical grade phosphoric acid was used to modify the pH of phosphate solutions. Both these reagents were purchased from Mallinckrodt.

For acetic acid determination, phthalic acid and cetyltrimethylammonium bromide (CTAB) were used as the UV-absorbing electrolyte and electro-osmotic flow modifier, respectively. Sodium acetate (equivalents of HOAc) was used to prepare the reference standard solutions and L-glutamic acid (L-Glu) was used as the internal standard (I.S.). These chemicals were purchased from Aldrich (Milwaukee, WI, USA).

All reference standard solutions, sample and electrolyte solutions for CE were prepared using distilled, deionized water.

2.3. Peptide sample

The peptide sample, bradykinin acetate, was purchased from Sigma (St. Louis, MO, USA).

3. Procedure

3.1. Peptide purity determination by HPLC

HPLC was performed under a typical gradient suitable for resolving peptides. Two solvents consisting of 0.1% (w/v) TFA in water, solvent A, and 0.1% (w/v) TFA in ACN, solvent B, were prepared to be used in the gradient elution. Sample solutions were prepared by dissolving bradykinin in A–B (95:5), 1.0 mg ml^{-1}. The flow-rate was adjusted to 1.0 ml min^{-1} and the system was equilibrated at 5% B. Aliquots of 10 or 25 μl were injected. After injection, 5% B was pumped isocratically for 10 min which was followed by gradient elution from 5 to 50% B over 30 min. The system was held at 50% B for 10 min before returning to the initial conditions. An interval of 30 min at 5% B was allowed for the system to re-equilibrate before subsequent injection. Detection was monitored at 215 nm UV and the column was thermostated at 24°C. All experiments were performed under the same conditions using both 300 and 90 Å pore size columns.

3.2. Peptide purity determination by CE

For purity determination by CE, a 32 cm×50 μm LPA coated capillary and 50 m*M* phosphate–phosphoric acid electrolytes of pH 2.5, 3.5, and 4.5 were used. The carousel housing as well as the capillary were maintained at ambient temperature. The capillary was first purged with water for 1.5 min and then with the electrolyte for 1.5 min before loading the sample, which was achieved by applying a pressure of 40 mbar for 6 s. The sample was electrophoresed at a voltage of 18 kV under positive polarity for 15 min and detection was monitored at 215 nm UV.

3.3. Counter ion determination by CE

The counter ion of bradykinin, acetic acid was determined using the validated method reported by Hettiarachchi and Ridge [15].

A 500 ml stock solution of L-Glu, 0.05 mg ml^{-1} was prepared for use as the I.S. solution. Seven reference standard solutions of HOAc (equivalents of NaOAc) were prepared by accurately weighing samples of NaOAc into separate 50-ml volumetric flasks and dissolving each sample in the I.S. solution. These solutions covered a range from 30 to 150% of the labeled value of 7.4% HOAc in the bradykinin sample. Five bradykinin acetate solutions were prepared by accurately weighing 1.0-mg portions into 1-dram vials and dissolving each in 2.0 ml of the I.S. solution (1 dram=3.697 ml).

CE was performed by using a 54 cm×50 μm fused-silica capillary. A solution of 5 m*M* sodium phthalate of pH 5.8, containing 5 m*M* CTAB was used as the UV-absorbing background electrolyte. The capillary was flushed with water followed by the electrolyte prior to each injection as described above. Injection was achieved by a pressure of 40 mbar for 6 s. All reference standard solutions and bradykinin sample solutions were electrophoresed at a voltage of 18 kV under negative polarity and monitored by indirect UV detection at 200 nm. Electrophoretic run times were as short as 5 min. The peak area (PA) ratios of the HOAc peak to the I.S. peak were

Table 1
Reference data for HOAc determination

Run No.	$W_{NaOAc\ (mg\ (50ml)^{-1})}$	$W_{HOAc\ (mg\ ml^{-1})}$	PA HOAc	PA I.S.	PA Ratio$_{(HOAc/I.S.)}$	Back-Calc HOAC$_{(mg\ ml^{-1})}$	% Deviation
1	1.754	0.025	22293	30282	0.736	0.026	4.113
2	2.964	0.042	37540	30769	1.220	0.042	−0.992
3	3.349	0.048	42569	31030	1.372	0.047	−1.991
4	4.662	0.067	57794	29388	1.967	0.066	−0.371
5	5.316	0.076	68614	30254	2.268	0.076	0.357
6	6.168	0.088	78398	30097	2.605	0.087	−0.993
7	7.517	0.108	100034	30795	3.248	0.109	0.851

$y = 30.4989x - 0.0610$, $R^2 = 0.9992$
Error = 1.38%

calculated for each electropherogram. When the PA ratios for the reference samples were plotted against their corresponding HOAc concentrations, the linear equation obtained for the curve was $y = 30.4989x - 0.0610$ with an error of 1.38%. Linear regression analysis of the data yielded an R^2 value of 0.9992. Data for the reference standard solutions are shown in Table 1.

Above linear equation for the reference curve was employed to calculate the amount HOAc in each bradykinin solution. Data for these solutions are given in Table 2.

4. Results and discussion

4.1. Peptide purity determination

Reversed-phase HPLC is widely used in peptide purification and analysis due to its high resolving power. Peptides are commonly eluted from reversed-phase columns using an aqueous mobile phase containing an ion pairing agent and an organic modifier. According to literature, C_{18} columns using TFA as the ion pairing agent and ACN as the organic modifier have been very popular and such systems have been increasingly used in peptide analysis.

In these experiments two types of reversed-phase columns, a 300 Å pore size (218TP54) and a 90 Å pore size (201HS54) C_{18}, from Vydac were used to determine the peptide purity. The chromatogram of bradykinin obtained using the 300 Å pore size C_{18} column is shown in Fig. 1. It shows a high degree of purity for bradykinin (>99.5%), with only 1 detectable impurity, a minor peak residing on the shoulder of the major peak. In comparison, the 90 Å pore size C_{18} showed improved resolution. With this column, as shown in Fig. 2, three partially resolved minor impurities appeared on both shoulders of the major peak. The major peak accounted for 99.4% of the total peak area. The 90 Å pore size column (201HS54) with a high surface area and carbon load is specially designed for small peptides (2–10 residues) that are basic and hydrophilic, as compared to the 300 Å pore size (218TP54) column. The results obtained are in agreement as expected, and therefore,

Table 2
Sample data for HOAc determination

Run No.	$W_{sample\ (mg)}$	$W_{sample\ (mg\ ml^{-1})}$	PA HOAc	PA I.S.	PA Ratio$_{(HOAc/I.S.)}$	Found(mg HOAc)	%HOAc
1	1.006	0.503	37733	30718	1.228	0.042	8.405
2	0.953	0.477	38697	31368	1.234	0.042	8.908
3	0.634	0.317	26127	31010	0.843	0.030	9.345
4	0.738	0.369	30330	31129	0.974	0.034	9.200
5	1.122	0.561	40319	29623	1.361	0.047	8.311
						Average 8.83±0.38%	

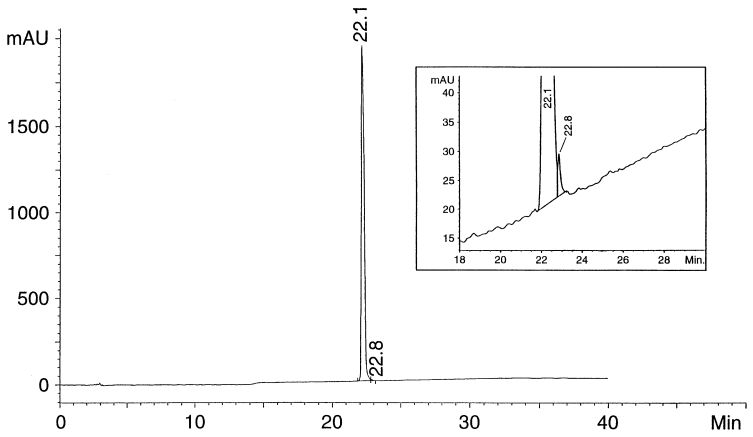

Fig. 1. HPLC separation of bradykinin using a 300 Å pore size Vydac C_{18} column, 250×4.6 mm.

the 90 Å pore size column is better in resolving small peptides from their closely associated impurities.

The feasibility of application of CE to perform peptide analysis was first demonstrated by Jorgenson et al., [16] in 1981. A major problem in early CE work was associated with sample adsorption on the capillary wall. As demonstrated first by Hjertén [17] in 1985, development of LPA coated capillaries eliminated sample adsorption and also electroendosmosis. Thereafter, use of a coated capillary and a low pH buffer became a very convenient method for peptide analysis by CE [18]. Reported methods on analysis of bradykinin [19–21] and similar peptides by CE also utilize coated capillaries and low pH buffers.

Adjustment of buffer pH is the most direct approach to optimize the resolution of peptides [22] in CE analysis. As demonstrated by Zhu et al., [20], Rickard et al., [23], and Landers et al., [24] the mobility, μ, of a peptide is governed by the relationship $\mu = kZM^{-2/3}$ where Z and M are charge and molecular mass, respectively, and k is a constant. To alter selectivity in peptide separations, changes in either charge or molecular mass must occur. Since molecular mass is essentially invariant (with the

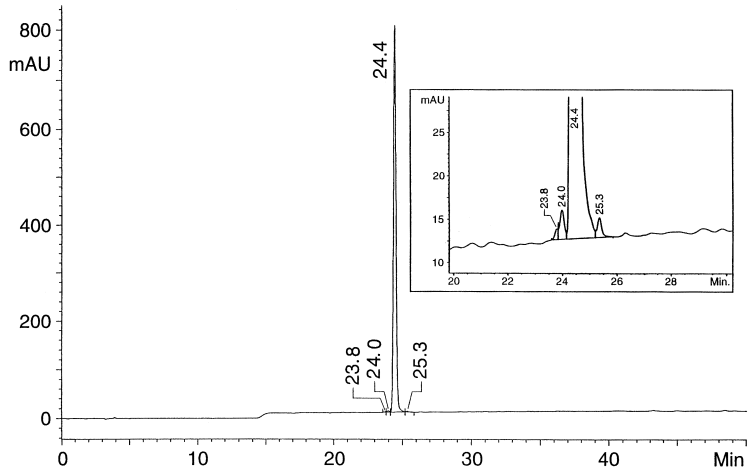

Fig. 2. HPLC separation of bradykinin using a 90 Å pore size Vydac C_{18} column, 250×4.6 mm.

negligible exception of gain or loss of protons), the parameter that can alter selectivity to the greatest extent is a change in the charge on the peptide. This is achieved most readily by altering the pH of the separation buffer, thereby directly affecting the extent of protonation of amine and ionization of the carboxylic moieties in the peptide. On the basis of these guidelines, an attempt was made to resolve the impurities of bradykinin by manipulating pH of the electrophoretic systems.

Typical electropherograms of bradykinin obtained with pH 2.5, 3.5, and 4.5 phosphate–phosphoric acid electrolytes are shown in Fig. 3a–c. Each figure indicates a high purity sample of bradykinin (99%). A remarkable separation of the impurities was observed when the pH was first increased to 3.5 and then to 4.5. Fig. 4a–c are reproductions of the same

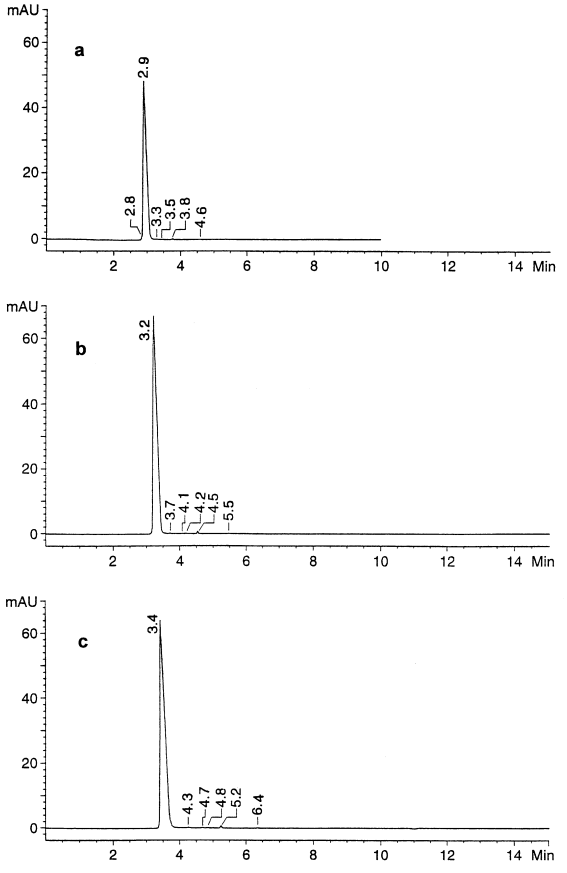

Fig. 3. CE separation of bradykinin using phosphate-phosphoric acid electrolytes of pH 2.5 (a), 3.5 (b) and 4.5 (c).

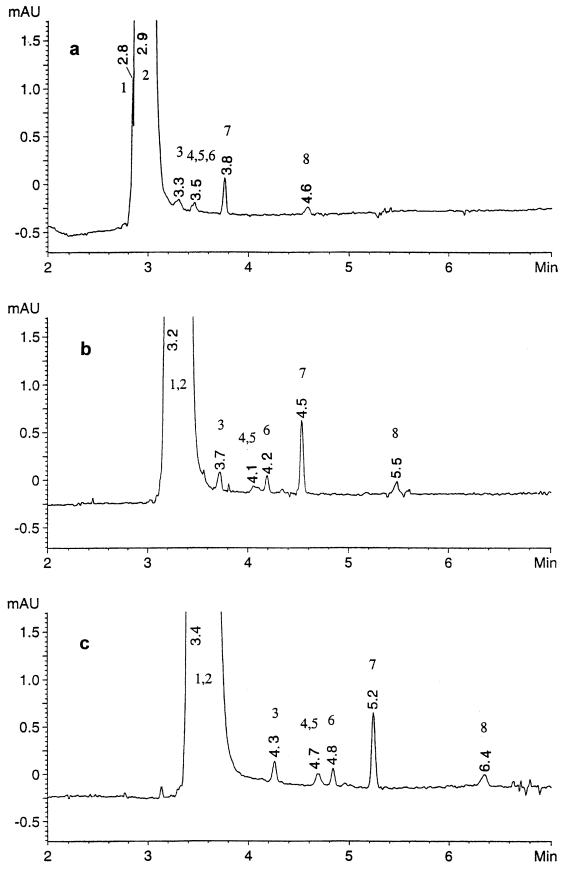

Fig. 4. (a–c) Expanded baselines of Fig. 3a–c, respectively, showing resolution of impurities.

electropherograms shown in Fig. 3a–c with expanded baselines. The major peak, bradykinin is numbered 2 in each figure and the peptide impurities are numbered 1 and 3–7. Impurity 1 was observed only with the pH 2.5 electrolyte. This impurity peak co-migrated with bradykinin under pH values of 3.5 and 4.5, however, resolution of the remaining impurity peaks, 3–8, improved as the pH was increased from 2.5 to 4.5. In contrast, none of these peaks were fully resolved by HPLC (Figs. 1 and 2).

4.2. Counter ion determination

Synthesizing basic peptides in the form of salts containing counter ions such as HOAc and TFA is a common practice. Unlike TFA, the presence of HOAc in therapeutic peptides may not cause any

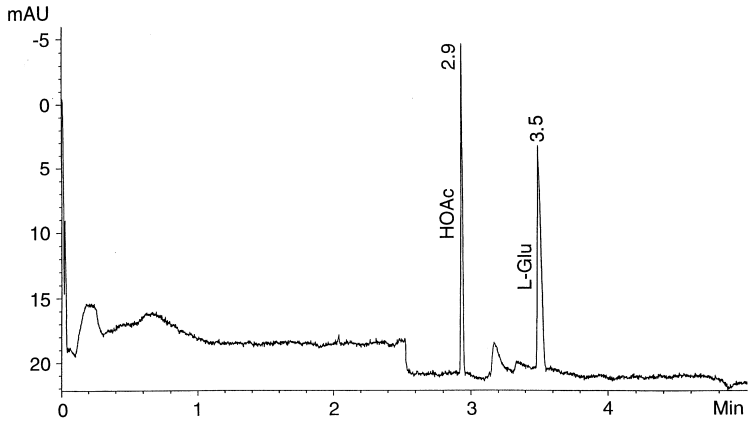

Fig. 5. Typical reference electropherogram showing HOAc (sodium acetate) and L-Glu (I.S.).

adverse toxic effects, however, its presence might lower the biological potency of the peptide. Therefore, accurate determination of the counter ion content in synthetic peptides is necessary. Determination of acetic acid in the bradykinin acetate sample was performed according to a validated method [15]. The data shown in Tables 1 and 2 indicate that the sample contains 8.8±0.4% HOAc (n=5), a result that is in reasonable agreement with the calculated value of 7.4% HOAc in a peptide that contains 1.5 moles of HOAc and 3.5 moles of water per mole of peptide as claimed by the manufacturer. Typical electropherograms of a reference acetic acid (sodium acetate) solution and a bradykinin acetate solution each containing the I.S. (L-Glu) are shown in Figs. 5 and 6.

5. Conclusions

In HPLC, utilization of a smaller pore size (90 Å) C_{18} column provides better resolution for a small peptide (typically 2–10 residues) from its closely associated impurities, in comparison to a larger pore size (300 Å) column.

For synthetic peptides that are purified by preparative HPLC, analysis must be performed using a different HPLC method than what was used in the purification, to prevent co-elution of closely related peptide impurities.

HPLC and CE are two powerful separation techniques available to the analytical chemist. Because these two techniques are based on different physicochemical properties, information derived from them

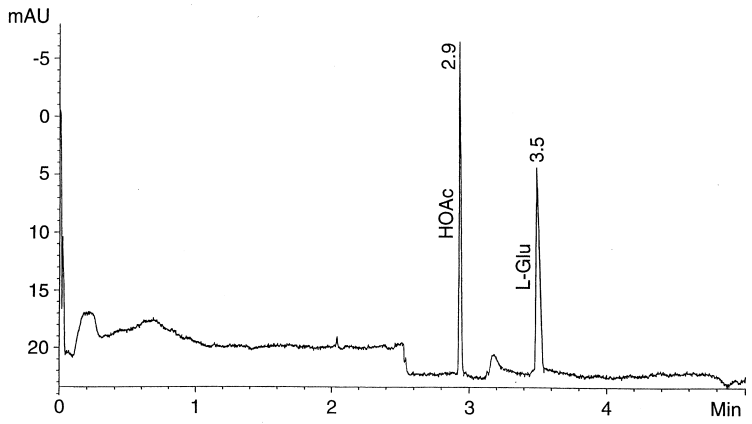

Fig. 6. Typical electropherogram of bradykinin acetate showing HOAc and L-Glu (I.S.).

is orthogonal. Therefore, incorporation of CE in peptide analysis in conjunction with HPLC increases the confidence of validity of analytical results. In addition, it also provides complementary results with better resolutions and separation efficiencies.

The CE assay that was performed to determine the content of acetic acid in bradykinin acetate is very efficient and accurate. The most attractive feature of the CE method is that each electrophoretic run needs only five min, an efficiency that cannot be paralleled by any other separation method.

References

[1] R.B. Merrifield, J. Am. Chem. Soc. 85 (1963) 2149.
[2] R.B. Merrifield, J. Am. Chem. Soc. 86 (1963) 304.
[3] R.B. Merrifield, Biochemistry 3 (1964) 1385.
[4] R.B. Merrifield, J.M. Stewart, Nature 207 (1965) 522.
[5] R.B. Merrifield, J.M. Stewart, N. Jernberg, Anal. Chem. 38 (1966) 1905.
[6] A.H. Brady, J.W. Ryan, J.M. Stewart, Biochem J. 121 (1971) 179.
[7] R.A. Boissonnas, St. Guttmann, P.-A. Jaquenoud, Helv. Chim. Acta 43 (1960) 1349.
[8] E.D. Nicolaides, H.A. De Wald, J. Org. Chem. 26 (1961) 3872.
[9] St. Guttmann, J. Pless, R.A. Boissonnas, Helv. Chim. Acta 45 (1962) 170.
[10] P.D. Grossman, J.C. Colburn, H.K. Lauer, R.G. Nielsen, R.M. Riggin, G.S. Sittampalam, E.C. Ricka, Anal. Chem. 61 (1989) 1186.
[11] V.E. Klushnichenko, D.M. Koulich, S.A. Yakimov, K.V. Maltsev, G.A. Grishina, I.V. Nazimov, A.N. Wulfson, J. Chromatogr. A 661 (1994) 83.
[12] R. Hynek, V. Kašicka, Z. Kucerová, J. Káš, J. Chromatogr. B 681 (1996) 37.
[13] V. Pacákova, J. Suchánková, K. Štulík, J. Chromatogr. B 681 (1996) 69.
[14] A. Vinther, S. Bjorn, H.H. Sorensen, H. Soeberg, J. Chromatogr. 516 (1990) 175.
[15] K. Hettiarachchi, S. Ridge, J. Chromatogr. A 817 (1998) 153.
[16] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[17] S. Hjertén, J. Chromatogr. 347 (1985) 191.
[18] R.M. McCormick, Anal. Chem. 60 (1988) 2322.
[19] Laboratories Application Note 9, Bio–Rad Labs., Hercules, CA, 1989.
[20] M. Zhu, D. Hansen, S. Burd, V. Huebner, P. Balasubramanian, A.J.C. Chen, Laboratories Bulletin No. 1482, Bio-Rad Labs, Hercules, CA, 1989.
[21] Bulletin 886, Supelco, Bellefonte, PA, 1994.
[22] P.D. Grossman, K.J. Wilson, G. Petrie, H.H. Lauer, Anal. Biochem. 173 (1988) 265.
[23] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197.
[24] J.P. Landers, R.P. Oda, J.A. Liebenow, T.C. Spelsberg, J. Chromatogr. A 652 (1993) 109.