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Research Article

Assay of bradykinin-related peptides in human body fluids using capillary electrophoresis with laser-induced fluorescence detection

A CE with LIF detection was developed for separation and determination of bradykinin (BK)-related peptides, such as BK, kallidin (Kal), and neurokinin A (NKA). BK-related peptides were derivatized with FITC prior to CE-LIF analysis. Sodium borate 10 mmol/L at pH 9.5 was selected as derivatization media in order to get the high efficiency. Three peptides were baseline-separated within 10 min by using 110 mmol/L sodium borate–sodium hydroxide solution at pH 10.0 as the running buffer. Concentration detection limits (S/N = 3) for BK, Kal, and NKA were 0.08, 0.5, and 0.2 nmol/L, respectively. Meanwhile we have also developed a simple, quick, and sensitive large-volume sample stacking (LVSS) technique for CE-LIF detection of BK, Kal, and NKA. By using this stacking technique, the detection limits (S/N = 3) for BK, Kal, and NKA were 0.02, 0.05, and 0.04 nmol/L, respectively. This method has been applied to the assay of human saliva and cerebrospinal fluid with satisfactory results.

Keywords:
Bradykinin-related peptides / Fluorescein isothiocyanate / Large-volume sample stacking / Laser-induced fluorescence
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1 Introduction

Bioactive peptides in body fluids have received more and more attention in recent years, because they are involved in the regulation of physiological processes at very low concentrations. Bradykinin (BK)-related peptides, such as BK, kallidin (Kal), and neurokinin A (NKA), belong to kinins, which are a family of peptide hormones that are responsible for a variety of very important physiological and pathophysiological effects. Their pharmacological effects include cardiovascular and algogenic actions, the latter being associated with increased capillary permeability, production of edema, and the initiation of pain and associated reflexes [1, 2]. Therefore, the trace amount endogenous peptides in biological tissues and fluids must be determined accurately so that their physiological effects and clinical pathology can be better understood.

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Abbreviations: BK, bradykinin; **CBQCA,** 3-(4-carboxy-benzoyl)-2-quinoline-carboxaldehyde; **CSF,** cerebrospinal fluid; **Kal,** kallidin; **LVSS,** large-volume sample stacking; **NBD-F,** 4-fluorine-7-nitro-benzo-oxadiazol; **NKA,** neurokinin A

For the determination of kinins, RIA or ELISA has often been employed for its high sensitivity [2–6]. However, the most obvious disadvantage of RIA is the safety concern of using radioactive species. In addition, RIA involves labor-intensive procedures aside from sample preparation and lengthy reaction times. Although ELISA do not use radioactive reagents, some intrinsic problems involved in immunoassay, such as cross-reaction and nonspecific interference of coexistent compounds with the binding of the analytes, still need to be resolved.

HPLC with UV or fluorescence detection has been described for determination of BK standard or BK spiked in biological fluids [7–9]. However, HPLC requires relatively large sample quantities for analysis, which can be problematic when handling biological fluids and the separation in HPLC was always time-consuming. It was concluded that UV detection is often inadequate for use in the detection of BK in biological samples. This is due to its poor sensitivity and the coexistence in other biological sample, which exhibits very similar UV spectra comprised of a large absorption band and increase the possibility of overlapping peaks.

CE has become a powerful tool for the separation and detection of peptide mixtures [10–12]. CE analysis of BK has also been employed with UV or LIF detection [13, 14]. CE offers several advantages over chromatographic techniques for the analysis of biological samples, such as high-speed and

high-efficiency separation, utilization relatively inexpensive and long lasting capillary columns, and consumes small volumes of sample and reagent. Since extremely small volumes (pL–nL) can be injected into the capillary easily, this results in improved component resolution and also a high mass sensitivity [15]. Unfortunately, the concentration sensitivity by using UV absorption is relatively poor when compared to HPLC methods, because the small injection volumes and short optical path-length (25–75 μm) used in most systems. In practice, the optical path-length can be increased by incorporating bubble, Z-shaped, or multi-reflection flow cells. These modifications generally yield a ten-fold path-length extension but are accompanied by a reduction in component resolution [16–20].

The LIF method affords concentration detection limit three orders of magnitude better than that of UV techniques [21, 22]. The Ar^+ laser with excitation at 488 nm and emission at 535 nm is the most popular and commercially available. Few peptides contain a native fluorophore, thus for CE-LIF detection most peptides need to be derivatized with a fluorescent probe [23–25]. Siri *et al*. [23] reported an automated large-volume sample stacking (LVSS) procedure to detect FITC-labeled BK and other peptides in the picomolar range. In this paper, we compared using of three derivatization reagents: 3-(4-carboxy-benzoyl)-2-quinoline-carboxaldehyde (CBQCA), 4-fluorine-7-nitrobenzo-oxadiazol (NBD-F), and FITC. A quantitative and sensitive CE-LIF method using FITC as precolumn derivatization reagent for simultaneous separation and detection of BK, Kal, and NKA (see Table 1) was developed. The method has been successfully applied to the assay of BK and Kal in human saliva and NKA in cerebrospinal fluid (CSF). Meanwhile we have also developed a simple, quick, and sensitive automated LVSS technique by CE-LIF detection for the detection of BK, Kal, and NKA. The detection limit of BK was similar to that of the work of Siri *et al*. [23].

Table 1. The BK-related peptides

Peptide	Abbreviation	Relative molecular mass
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	BK	1240.4
Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	Kal	1032.2
His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met	NKA	1133.3

2 Materials and methods

2.1 Chemicals

BK, Kal, NKA, FITC, and NBD-F were obtained from Sigma (Saint Louis, MO, USA); CBQCA was obtained from Molecular Probes (Eugene, USA); ACN (HPLC grade), sodium hydroxide, and sodium borate (analytical grade) were from Shanghai Chemical Factory (Shanghai, China). Potassium cyanide (analytical grade) was obtained from Fushan Chemical Factory (Fushan, China). The water was prepared using a Millipore (Bedford, MA, USA).

2.2 Instrumentation

A P/ACE MDQ CE instrument equipped with LIF detector (Beckman, Fullerton, CA, USA) was used for the experiments. The LIF detector used an argon ion laser for excitation at 488 nm and emission at 535 nm. Data collection, processing, and analysis were performed with system 32-Karat software (Beckman) and recorded on an IBM-compatible personal computer. Electrophoresis was performed in a bare fused-silica capillary (Rui-feng, Yongnian, Hebei, China), 60 cm (effective length 50 cm) × 75 μm id. C_{18}-ODS cartridges (500 mg/6 mL) were obtained from Agilent.

For FITC derivatization reaction, 10 μL standard peptides mixed solution was reconstituted in 100 μL sodium borate buffer (10 mmol/L, pH 9.5). Then 10 μL FITC (in acetone) and 80 μL ACN were added to the solution. The derivatized solution was kept in the dark for 16 h. The obtained solution was diluted either to 10 or 100 times prior to injection, 110 mmol/L sodium borate was used as running buffer, which was adjusted to pH 10.0 by addition of 1 mol/L sodium hydroxide. The buffer solution was filtered through a 0.45 μm pore-size membrane filter and degassed before use. At the start of each run, the capillary was rinsed with 0.1 mol/L sodium hydroxide for 1 min, with Millipore water 1 min, followed by the running buffer for 3 min.

2.3 Sample preparation and derivatization

CSF samples were collected from the patients of brain aneurysm subarachnoid hemorrhage and hypophyseal adenoma (provided by the Department of Neurology, Fuzhou General Hospital). The procedures used for obtaining kinins from saliva or CSF samples include deproteination and SPE using C_{18}-ODS cartridges. Two milliliters of saliva or CSF was collected in a 10 mL polyethylene centrifuge tube, and 2 mL of ACN was added to the sample. The sample mixture was centrifuged at 5000 × *g* for 5 min. The supernatant was passed through the C_{18}-ODS cartridge. The C_{18}-ODS cartridge was washed with 3 mL water and the tested peptides were eluted using 3 mL of 50% v/v ACN/1% v/v aqueous acetic acid. The eluate of saliva sample was dried by N_2 gas and reconstituted in 200 μL derivatization buffer (10 mmol/L of sodium borate solution with pH 9.5). The eluate of CSF sample as dried by N_2 and reconstituted in 100 μL derivatization buffer. Then 10 μL of the solution was taken for derivatization.

3 Results and discussion

3.1 Optimization of fluorescence derivatization conditions

Three labeling reagents, such as CBQCA, NBD-F, and FITC, have been tried to be used for precolumn derivatization of BK, Kal, and NKA. Figure 1 shows that FITC is the most suitable and sensitive reagent for labeling BK, Kal, and NKA with 488 nm as the excitation wavelength and 535 nm as the emission wavelength. Although FITC has intrinsic fluorescence, the peaks of FITC appear after 9.5 min. The maximum excitation and emission wavelength of derivants formed by FITC and analytes matched those of the used Ar^+ laser, therefore FITC was selected as the label reagent for BK, Kal, and NKA.

Several effect factors on the labeling, such as buffer pH, concentration of buffer, reaction time, and the concentration of FITC, were investigated in detail to get the maximum labeling efficiency. The effect of pH on the labeling efficiency was investigated in the range of 7.0–10.0, and the results show that the peak heights of BK, Kal, and NKA are increased with the increasing of pH before pH 9.5, after that the peaks are decreased. Therefore, sodium borate buffer with pH 9.5 was selected as the derivatization buffer.

The influence of the concentrations of sodium borate buffer (pH 9.5) ranged from 5 to 60 mmol/L on fluorescent intensity were studied, and the result shows that the maximum reaction yield was achieved at a sodium borate concentration of 10 mmol/L. Thus, 10 mmol/L of sodium borate solution was used for subsequent experiments.

By comparing the peak heights of derivatives at different concentrations of FITC, the result showed that high concentration and excessive amount of FITC was required to obtain the high efficiency for labeling. However, when concentration of FITC was too high, the high background of excess FITC would destroy the CE separation. While using low concentration of FITC, such as 1×10^{-6} mol/L, fluorescence signals for the derivatized analyte were much weaker. FITC (6×10^{-6} mol/L) was found to be optimal.

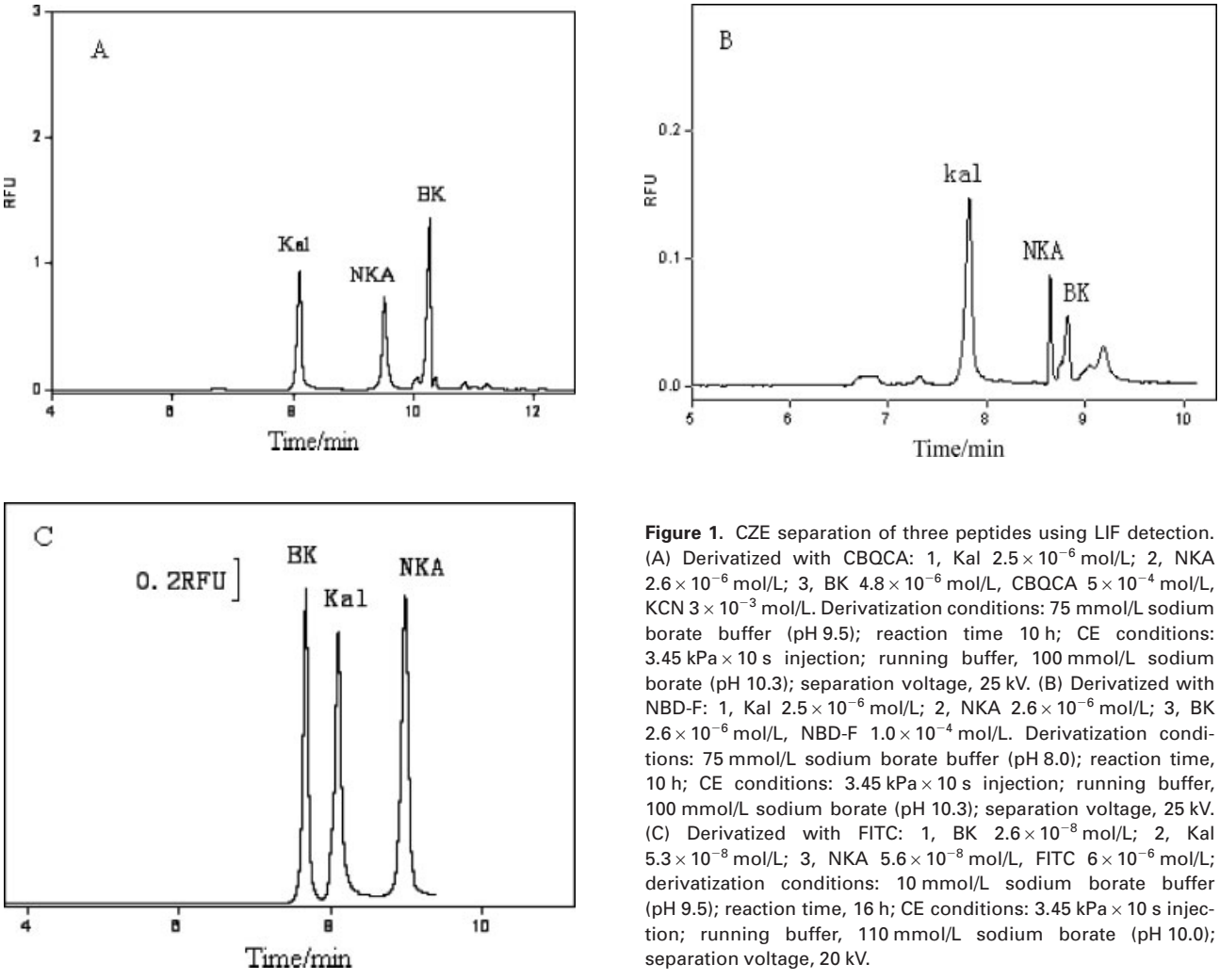

Figure 1. CZE separation of three peptides using LIF detection. (A) Derivatized with CBQCA: 1, Kal 2.5×10^{-6} mol/L; 2, NKA 2.6×10^{-6} mol/L; 3, BK 4.8×10^{-6} mol/L, CBQCA 5×10^{-4} mol/L, KCN 3×10^{-3} mol/L. Derivatization conditions: 75 mmol/L sodium borate buffer (pH 9.5); reaction time 10 h; CE conditions: 3.45 kPa × 10 s injection; running buffer, 100 mmol/L sodium borate (pH 10.3); separation voltage, 25 kV. (B) Derivatized with NBD-F: 1, Kal 2.5×10^{-6} mol/L; 2, NKA 2.6×10^{-6} mol/L; 3, BK 2.6×10^{-6} mol/L, NBD-F 1.0×10^{-4} mol/L. Derivatization conditions: 75 mmol/L sodium borate buffer (pH 8.0); reaction time, 10 h; CE conditions: 3.45 kPa × 10 s injection; running buffer, 100 mmol/L sodium borate (pH 10.3); separation voltage, 25 kV. (C) Derivatized with FITC: 1, BK 2.6×10^{-8} mol/L; 2, Kal 5.3×10^{-8} mol/L; 3, NKA 5.6×10^{-8} mol/L, FITC 6×10^{-6} mol/L; derivatization conditions: 10 mmol/L sodium borate buffer (pH 9.5); reaction time, 16 h; CE conditions: 3.45 kPa × 10 s injection; running buffer, 110 mmol/L sodium borate (pH 10.0); separation voltage, 20 kV.

The reaction time for derivatization was investigated by comparison peak heights of derivatives in various time intervals. The results showed that the derivatization reaction could be completed to get a maximum peak height in 16 h at room temperature, and could be remained at this level about 40 h. In subsequent experiments, 24 h was used as the analytes derivatization time.

3.2 Optimization of separation conditions

Different buffer systems, such as phosphate, borate, carbonate, and citrate with different pH were examined as the running buffer for the separation of BK, Kal, and NKA. The results showed that sodium borate buffer was the optimal running buffer due to its higher separation efficiency. Sodium borate buffer (110 mmol/L) solution was used as the running buffer to examine the effect of pH on the separation at a separation voltage of 20 kV. The result indicates when pH is lower than 9.6, BK and Kal cannot be baseline separated, increasing of buffer pH would result in an increased resolution between BK and Kal and a longer separation time. We also investigated the influence of running buffer pH on peak heights of analytes. Three analytes had highest peak heights at pH 10.0, when pH was higher than 10.0, peak heights would decrease with increasing of pH.

The effect of different running buffer concentration (50, 75, 100, 110, 150 mmol/L) on the separation of three analytes was also studied at pH 10.0 and an applied voltage of 20 kV. When running buffer concentration was less than 110 mmol/L, three analytes could not be baseline-separated. Higher concentration resulted in longer analysis time and higher Joule heat. In consideration of selectivity, sensitivity, resolution, and analysis time, 110 mmol/L sodium borate with pH 10.0 was selected as running buffer.

Under the optimum derivatization and separation conditions, three analytes were baseline-separated within 10 min (see Fig. 1C).

3.3 Linear regression equation and detection limit

The linear response range and the detection limits for BK, Kal, and NKA under the optimal conditions are listed in Table 2. The detection limit was defined as the concentration based on the S/N of 3. The precision for this method was evaluated by measuring the repeatability of migration times and peak heights for each analyte. The precisions for the peak heights of BK, Kal, and NKA was in the range of 3–6% ($n = 5$, 2.6×10^{-8} mol/L for BK, 5.3×10^{-8} mol/L for Kal and 5.6×10^{-8} mol/L for NKA), and the RSD for migration time was less than 3% ($n = 5$), which indicated that the proposed method has excellent precision.

3.4 LVSS using the EOF pump and backpressure

Sample stacking is an inherent and exclusive feature of CE; it may take place when the conductivity of the sample is smaller than that of the buffer. In large-volume sample injection, in order to achieve the sample stacking without sacrificing the high resolution of CE, the sample matrix should be removed without causing loss of the stacked sample prior to separation. In LVSS using the EOF pump, the electrophoretic mobility of the target ions must be in the direction opposite to that of the EOF in order that cations or anions could be focused at the rear or front of the sample zone [26]. In this paper, pH 10.0, 110 mmol/L and pH 7.5, 10 mmol/L sodium borates were selected as BGE and the sample media, respectively. The pH between the sample media and the background solution is discontinuous, which causes an alteration in the analytes's mobility as they pass through the boundary. The sample zone is neutral, creating a region of low conductivity where the analytes migrate quickly until they reach the boundary with background buffer and slow down and subsequently stack. The sample solutions were injected in the hydrodynamic mode by large volume (10.35 kPa, 67.5 s). After sample injection, a negative voltage (−15 kV) and a backpressure (1.38 kPa) were applied to affect the sample matrix removal. When the current reached 90% of the original value, the negative voltage and backpressure were demolished, and a 20 kV forward voltage was then used for subsequent separation. The comparison in peak heights improvement in detector response in terms of peak height, more than ten-fold of sensitivity could be obtained. Figure 2 shows the typical electropherograms for the three analytes with LVSS and without LVSS. The results indicated the pmol/L level of detection limit for above three BK-related peptides can be obtained by using CE-LIF plus LVSS. The linear regression equations and detection limits with LVSS are shown in Table 3.

Table 2. Linear regression equations and detection limits

Peptide	Linear regression equation[a)]	Correlation coefficient	Linear range (mol/L)	Detection limit (nmol/L)
BK	$Y = 5085.3X + 550.68$	0.994	1.3×10^{-10}–6.5×10^{-8}	0.08
Kal	$Y = 2259.1X - 1889.9$	0.998	1.0×10^{-9}–4.0×10^{-7}	0.5
NKA	$Y = 2467.0X + 95.05$	0.998	2.8×10^{-10}–1.4×10^{-7}	0.2

a) *Y,* peak height; *X,* concentration in nmol/L.

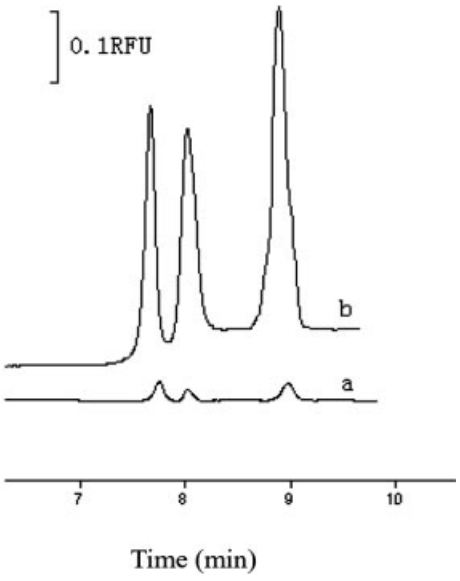

Figure 2. Electropherogram of three peptides without and with LVSS. (a) Without LVSS, conditions are the same as in Fig. 1C. (b) With LVSS, the sample solutions were injected in the hydrodynamic mode by large volume (10.35 kPa, 67.5 s). After sample injection, a negative voltage (−15 kV) and a backpressure (1.38 kPa) were applied to affect the sample matrix removal. Separation voltage: 20 kV. BK = 1.3 nmol/L; Kal = 2.6 nmol/L; NKA = 2.8 nmol/L.

3.5 Analytical application to real human saliva and CSF samples

The saliva samples were pretreated as described in Section 2.2. A typical electropherogram of human saliva sample is shown in Fig. 3. The concentration of BK in two saliva samples was in agreement with previously published values [7]. The levels of BK and Kal in saliva samples are shown in Table 4. The recoveries of these two real samples have been also examined, and the results are acceptable.

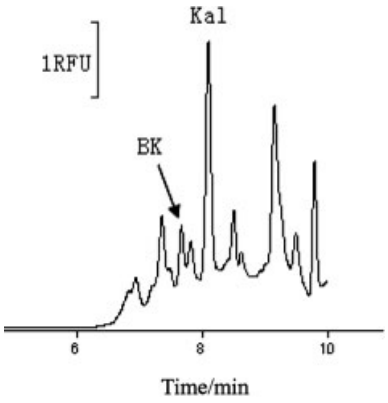

Figure 3. Electropherogram of the human saliva sample. Derivatization conditions and CE conditions are the same as in Fig. 1C.

The human CSF samples were pretreated as described in Section 2.2. NKA levels in CSF of two different patients were determined, and the results are shown in Table 5. A typical electropherogram of CSF sample is shown in Fig. 4. The results show that the CE-LIF method is valuable for the determination of NKA in CSF samples.

4 Concluding remarks

CZE with LIF detection has been used to separate three BK-related peptides which were precapillary derived by FITC.

Table 3. Linear regression equations and detection limits with LVSS

Peptide	Linear regression equation[a)]	Correlation coefficient	Linear range (mol/L)	Detection limit (nmol/L)
BK	$Y = 2303.2X + 17.998$	0.9999	$5.0 \times 10^{-11} - 1.3 \times 10^{-8}$	0.02
Kal	$Y = 1008.3X + 18.50$	0.9995	$1.0 \times 10^{-10} - 2.6 \times 10^{-9}$	0.05
NKA	$Y = 1369X - 774.25$	0.9999	$1.0 \times 10^{-10} - 2.8 \times 10^{-9}$	0.04

a) *Y*, peak height; *X*, concentration in 10^{-10} mol/L.

Table 4. Determination of BK and Kal in human saliva samples

Species	Peptide	Concentration (nmol/L ± SD; *n* = 3)	Added (nmol/L)	Founded (nmol/L)	Recovery (%)	RSD (%; *n* = 3)
Saliva (1)	BK	42.72 ± 0.05	13.00	11.62	89.4	3.3
	Kal	305.45 ± 0.04	13.00	12.54	96.5	3.5
Saliva (2)	BK	22.63 ± 0.06	13.00	11.89	91.5	2.8
	Kal	276.56 ± 0.08	13.00	12.80	98.5	4.2

Table 5. Determination of NKA in human CSF samples

Species	Peptide	Concentration (nmol/L ± SD; $n = 3$)	Added (nmol/L)	Founded (nmol/L)	Recovery (%)	RSD (%; $n = 3$)
CSF (1)	NKA	2.31 ± 0.04	2.80	2.68	95.7	4.5
CSF (2)	NKA	2.24 ± 0.03	2.80	2.72	97.1	3.2

CSF (1) is derived from a patient suffering from brain aneurysm subarachnoid hemorrhage; CSF (2) is derived from a patient with hypophyseal adenoma.

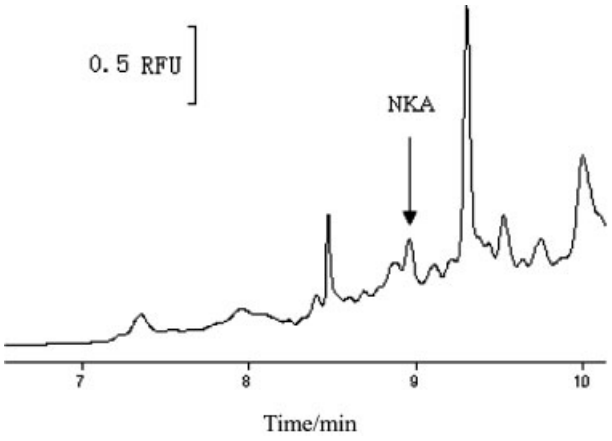

Figure 4. Electropherogram of the human CSF sample. Derivatization conditions and CE conditions are the same as in Fig. 1C.

Under the optimum conditions, three peptides were baseline-separated within 10 min. The method has been successfully applied to the assay of BK and Kal in human saliva and NKA in CSF. Meanwhile we have also developed a simple, quick, and sensitive LVSS technique for CE-LIF detection for BK, Kal, and NKA, the experiments showed that pmol/L level of detection limit for BK-related peptides can be obtained by using CE-LIF plus LVSS.

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The authors have declared no conflict of interest.

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