Published in final edited form as:

Anal Biochem. 2009 October 1; 393(1): 105–110. doi:10.1016/j.ab.2009.06.012.

Quantification of carnosine- related peptides by microchip electrophoresis with chemiluminescence detection

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Abstract

A microchip electrophoresis (MCE) method with chemiluminescence (CL) detection was developed for the determination of carnosine-related peptides including carnosine, homocarnosine and anserine in biological samples. A simple integrated MCE-CL system was built to perform the assays. The highly sensitive CL detection was achieved by means of the CL reaction between hydrogen peroxide and N-(4-aminobutyl)- N-ethylisoluminol-tagged peptides in the presence of adenine as a CL enhancer and Co²⁺ as a catalyst. Experimental conditions for analyte labeling, MCE separation, and CL detection were studied. MCE separation of the above said three peptides took less than 120 s. Detection limits (S/N=3) of 3.0×10^{-8} , 2.8×10^{-8} and 3.4×10^{-8} M were obtained for carnosine, anserine and homocarnosine, respectively. The present MCE-CL method was applied for the determination of carnosine, anserine and homocarnosine in human cerebrospinal fluid (CSF) and canine plasma. Homocarnosine was detected at the μ M level in the CSF samples analyzed while the levels of carnosine and anserine in these samples were below the detection limit of the assay. Interestingly, both carnosine and anserine were detected in the canine plasma samples, but not homocarnosine.

Keywords

Microchip electrophoresis; chemiluminescence detection; peptide analysis; N-(4-Aminobutyl)-N-ethylisoluminol; carnosine-related peptides; human cerebrospinal fluid; canine plasma

Carnosine-related peptides including carnosine (Car), homocarnosine (Hcar), and anserine (Ans) are a group of histidine-containing small peptides that widely occur in vertebrates [1, 2]. Over the past years, studies demonstrated that these peptides were involved in important biological roles such as antioxidant action, chelation of heavy metals, and modulation of enzyme activities [3,4]. It was also shown that the abnormality of their metabolism in the nervous system might be related to certain pathological conditions including Huntington's disease [5].

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Several analytical methods based on high performance liquid chromatography (HPLC) were reported for the determination of carnosine-related peptides present in biological samples [4, 6–8]. HPLC methods have good selectivity. However, HPLC has shortcomings including long analysis time and limited column lifetime. A capillary electrophoresis (CE) method with laser induced fluorescence detection was also developed for quantifying these peptides in meat and human cerebrospinal fluid (CSF) samples [9]. This method had detection limits of 4.7, 4.4, and 3.9 nM for carnosine, anserine, and homocarnosine, respectively. However, the CE-LIF system required sophisticated and expensive instrumentation. Microchip electrophoresis (MCE), as a miniaturized version of classical CE, has recently attracted considerable interest for its potential of miniaturization and integration of an entire chemical or biological analysis process on a single device. MCE has many advantages compared with HPLC and CE, which include higher separation efficiency, shorter run time, lower sample and reagent consumption, and ease of integration and automatization [10–13].

Chemiluminescence (CL) detection is a highly sensitive detection scheme. In addition, it is simple in instrumental setup and low in costs for operation and maintenance. Since a CL detector can be easily miniaturized, CL detection is particularly well suited for coupling with MCE separations to perform sensitive and selective MCE-CL bioassays. Over the past years, several MCE-CL methods were reported. Mangru and Harrison performed an interesting study on CL detection following MCE to monitor horseradish peroxidase (HRP) and fluorescein-conjugated HRP using the luminol CL system [14]. Tsukagoshi et al. developed a MCE system with CL detection for assaying dansyl amino acids, human serum albumin and immunosuppressive acidic protein [15,16]. However, since the microfluidic channels were extremely small, the sensitivity of these MCE-CL assays fell mostly in the range from 10^{-5} ~ 10^{-7} M (limit of detection, LOD).

In this work, we report on the development of a rapid and sensitive MCE-CL method for the determination of small peptides present in biomedical samples. Carnosine-related peptides were taken as the model analytes. To make the peptides CL detectable with high sensitivity, labeling of the peptides with N-(4-Aminobutyl)-N-ethylisoluminol (ABEI) was performed prior to the MCE separation. ABEI is a derivative of isoluminol and highly chemiluminescent upon being oxidized. It has been used to tag biogenic amines for HPLC [17], and pre-column CL labeling with ABEI has also been used in CL detection following CE separations of arginine, glycine and biogenic amines [18,19]. We reported recently the development of a MCE-CL method based on pre-column CL labeling with ABEI to determine biogenic amine [20]. An big advantage of using pre-column labeling technique is to avoid adding the CL reagent into the MCE running buffer, further reducing the background noise of CL detection. In addition, a CL enhancer (i.e. adenine) and a catalyst (i.e. Co^{2+}) were used to further improve the MCE-CL assay sensitivity. Use of adenine and Co^{2+} as enhancer and catalyst in MCE-CL assays have not been reported before. The MCE-CL method has the advantages of being fast, convenient, sensitive, and reproducible. The use of a microfluidic device coupled with CL detection for peptide analysis with a detection limit at the 10^{-8} M level was demonstrated for the first time in this work.

Materials and methods

Chemicals and solutions

Car, Ans, Hcar, adenine, glutathione (GSH), taurine (Tau), γ -amino-n-butyric acid (GABA), 20 protein amino acids, biogenic amines, and N,N'-Disuccinimidyl-carbonate (DSC) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). ABEI was purchased from Fluka (Buchs, Switzerland). Hydrogen peroxide (H_2O_2) was obtained from Taopu Chemicals (Shanghai, China). Sodium dodecyl sulfate (SDS) was provided by Shanghai Reagents (Shanghai, China). All other chemicals used in this work were of analytical grade. Water

purified by a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA) was used throughout the work. The electrophoretic buffer was 15 mM borate buffer (pH 9.8, adjusted with an 1M NaOH solution) containing 1.0 mM Co^{2+} , 1.0 mM adenine and 35 mM SDS. The CL oxidizer solution was 40 mM sodium bicarbonate buffer (pH12.5, adjusted with 1 M NaOH solution) containing 110 mM H_2O_2 . Stock solutions of ABEI and DSC were prepared in methanol and acetonitrile, respectively. A stock solution of peptides was prepared in water and diluted with water as needed. All solutions were filtered through 0.22 μm membrane filters before use.

MCE-CL System

The glass /PDMS hybrid microchip assembly was mounted on the X–Y translational stage of an inverted microscope (Olympus CKX41) that also served as a platform of CL detection. CL signal was collected by means of a microscope objective. After passing a dichroic mirror and a lens, CL photons were detected by a photomultiplier (PMT, Hamamatsu R105). The PMT was mounted in an integrated detection module including an HV power supply, a voltage divider, and an amplifier. The output signal of PMT was recorded and processed with a computer using a Chromatography Data System (Zhejiang University Star Information Technology, Hangzhou, China). A multi-terminal high voltage power supply, variable in the range of 0–8000 V (Shandong Normal University, Jinan, China), was used for sample loading and MCE separation. The inverted microscope was placed in a dark box.

Microchip Fabrication

A double T glass/PDMS hybrid microfluidic chip was used in this work. The schematic layout of the chip is illustrated in Fig. 1. Fabrication of the microchip is described as following. First, the glass substrate with microchannels was fabricated through standard photolithography, wet chemical etching techniques [21,22]. Sylgard 184 PDMS pre-polymer (Dow Corning, Midland, MI, USA) was mixed thoroughly with its curing agent at 10:1, w/w, and then degassed by vacuum pump. The mixture was cured against the As–Ga mold at 80 °C for 2 h. After the replica was peeled from the mold, holes were punched as reservoirs. The obtained glass substrate and PDMS cover plate were ultrasonically cleaned with acetone, methanol and water for 25 min, respectively. And then they were dried under an infrared lamp. After being exposed to UV light (6W, mercury lamp) from the distance of 3 cm for 3 h, the PDMS cover was bonded to the glass substrate. Microchannels measured 65 μm wide by 25 μm deep for sample introduction, separation and waste delivery, 250 μm wide by 25 μm deep for oxidizer introduction and CL detection, respectively. All reservoirs were 4.0 mm in diameter and 1.5 mm deep. The channel between reservoir S and SW was used for sampling, the channel between B and BW was used for the separation and the channel between R and BW was used for the oxidizer introduction. The join-point of the oxidizer introduction channel with the separation channel was used for the collection of CL.

Samples preparation

The human CSF samples were collected from patients with various neurological condition: (i) cephalitis, (ii) brain tumor, and (iii) surgical brain damage. A portion (400 μL) of a CSF sample was diluted with ice-cold 800 μL of acetonitrile and shaken vigorously for 5 minutes to precipitate proteins. After centrifuging at 16,000 rpm for 10 min, supernatant was transferred into 1.5 ml vial and dried with N_2 stream. Residue was re-dissolved in 20 μL of 20 mM borate buffer at pH 9.0. The solution was kept at 4 °C till analysis.

Canine plasma samples were obtained from three healthy dogs. The same procedure as stated above for sample preparation was used.

CL-labeling

To label the samples for CL detection, 25 μL of a 5 mM ABEI solution was added to an equal volume of 10 mM DSC solution. The mixture was allowed to react at room temperature for 2 h. The ABEI–DSC solution was then added to 10 μL sample solution. After mixing, the mixture was allowed to stand at room temperature for 2 h. The labeled sample was diluted appropriately with running buffer prior to loading if necessary.

MCE separation and CL detection

After the microchannels were rinsed sequentially with 0.1 M NaOH, water and electrophoretic buffer for 5 min each, reservoirs B, S, SW and BW were filled with the electrophoretic buffer, reservoir R filled with oxidizer solution. Vacuum was applied to reservoir BW in order to fill the separation channel with the electrophoresis buffer. Then, the electrophoretic buffer solution in reservoir S was replaced with sample solution. For loading the sample solution, a set of electrical potentials were applied to the five reservoirs: reservoir S at 750 V, reservoir B at 240 V, reservoir BW at 320 V, reservoir SW at grounded, and reservoir R floating. The sample solution was transported from reservoir S to SW in pinched mode. After 20 s, potentials were switched to reservoir B, S, SW and R at 2800, 1700, 1700 and 550 V, respectively, while reservoir BW was grounded for separation and detection.

Results and discussion

CL labeling of carnosine peptides

In this work, ABEI was used for pre-column CL labeling of peptides for subsequent MCE-CL assays. To achieve an effective labeling, ABEI was first coupled with DSC to form ABEI–DSC, which then reacted with peptides. Labeling reaction conditions including the molar ratio of ABEI–DSC to peptide and reaction time were investigated. The results showed that a molar ratio of ABEI–DSC to peptide of greater than 10 was needed. At room temperature, a maximal labeling yield was achieved at a reaction time of longer than 2 h.

CL detection of ABEI-tagged peptides

CL detection conditions were studied to obtain a maximal CL intensity (peak height). The tests were carried out by assaying a mixture of peptides at a concentration of 1.0×10^{-6} M each under varying conditions. H_2O_2 was used as oxidizer and its concentration affected greatly the CL intensity. Oxidizer solutions containing H_2O_2 at concentrations ranging from 60 to 120 mM were tested. The results indicated that the CL intensity first increased and then decreased with the increase in H_2O_2 concentration. The maximal CL signal was obtained at 90 mM H_2O_2 . It was noticed that the CL reaction between H_2O_2 and ABEI-tagged peptides was catalyzed by Co^{2+} . Effects of Co^{2+} concentration on the CL intensity were examined in the range of 0.4 – 1.2 mM. The results are illustrated by Fig. 2. As can be seen, the CL emission increased with the increase in Co^{2+} concentration till it reached a stable CL intensity at a Co^{2+} concentration of greater than 1.0 mM. It was also found that the CL emission of ABEI-tagged peptide- H_2O_2 - Co^{2+} system was enhanced in the presence of enhancers. Five different enhancers including adenine, gallic acid, rhodanine, xanthine, and benzothiazole were tested. The results indicated that adenine exhibited the strongest enhancing effect. Thus, adenine was chosen as the enhancer. Effect of adenine concentration on the CL intensity was investigated in the range of 0 – 1.2 mM. As shown in Fig. 3, maximum light emission occurred at 1.0 mM. It is worth noting the CL intensity was nearly 10 times higher than that without adenine.

MCE of ABEI-peptides

In this work, two separation modes, i.e. capillary zone electrophoresis and micellar electrokinetic chromatography (MEKC), were tested for the separation of ABEI - labeled

peptides. Our attempt to separate the three peptides by capillary zone electrophoresis in borate buffer failed due to their similarity in chemical structure. Therefore, SDS-mediated MECK was used for the separation. It was found that the introduction of SDS into the separation system significantly improved the resolution (i.e. $R_s = 2(t_2 - t_1) / (w_1 + w_2)$). The best separation was obtained with a running buffer at pH 9.8 containing 35 mM SDS. Separation voltage also affected the resolution. It was examined in the range of 2100–3000 V. The results showed that migration times and the resolution decreased with the increase in separation voltage. Considering both the analysis time and resolution, 2800 V was chosen as the separation voltage.

Based on the results mentioned above, the optimum separation and CL detection conditions were as following: running buffer was 15 mM borate buffer (pH 9.8) containing 1.0 mM Co^{2+} , 1.0 mM adenine and 35 mM SDS. The oxidizer solution was 40 mM sodium bicarbonate buffer (pH 12.5) containing 90 mM H_2O_2 . And the separation voltage was fixed at 2800 V. Under this optimum condition, a mixed standard solution containing 8.0×10^{-7} M peptides was analyzed, Fig.4 shows the electropherogram. As can be seen, the three peptides were separated completely within 120 s.

Interference study

Many endogenous amino acids and biogenic amines exist in biological samples. They react with ABEI-DSC to form ABEI derivatives, which may interfere with the determination of the peptides. Therefore, the influence of co-existing compounds such as γ -amino-n-butyric acid (GABA), dopamine (DA), noradrenalin (NE), agmatine (Agm), octopamine (Oct) and 20 protein amino acids were investigated. Fig.5 shows the electropherogram obtained from the separation of a mixture containing the above-mentioned compounds and carnosine peptides (i.e. Car, Hcar and Ans). As can be seen, Car, Hcar and Ans were well separated from the amino acids and amines included in the tested, which suggested that none of these endogenous amino acids and biogenic amines would interfere with the determination of Car, Hcar and Ans in biological samples.

Linearity, detection limit and reproducibility

Seven-point calibration curves were prepared by analyzing a series of peptide standards under the selected MCE-CL conditions. The results are shown in Table 1. Peptide concentration to the peak height showed a good linearity in the range of 6.0×10^{-8} ~ 1.5×10^{-5} M for all peptides, and the correlation coefficients ranged from 0.9927 to 0.9991. Detection limits ($S/N=3$) were estimated to be 3.0×10^{-8} M for Car, 2.8×10^{-8} M for Hcar, and 3.4×10^{-8} M for Ans, respectively. For the reproducibility, a standard solution containing Car, Hcar and Ans at 5.0×10^{-7} M each was analyzed seven times. The results showed that the RSDs of the migration time and peak height were lower than 1.8 and 3.5%, respectively.

Quantification of carnosine peptides in human CSF

Three CSF samples from patients of cephalitis, brain tumor and surgical brain damage were analyzed. A typical electropherogram from the analyses is shown in Fig.6A. As can be seen, Hcar was detected at the μM level, while Car and Ans were not detectable. To verify the peak identification, authentic Hcar was added to this sample, and then the spiked sample was again analyzed. The electropherogram obtained is shown in Fig.6B. The results from sample analysis are summarized in Table 2. The recovery of the assay was studied by analyzing human CSF samples spiked with standard Hcar solution. The results are also shown in Table 2. The recoveries of Hcar in CSF analysis were found to be in the range of 92.8–104.3%. The results indicated that the present MCE-CL method is useful for the determination of Hcar in CSF samples.

Quantification of carnosine peptides in canine plasma

To further evaluate the applicability of the MCE-CL method proposed in this work, another biological sample, canine plasma, was analyzed. A typical electropherogram from the analyses is shown in Fig. 7A. For peak identification, the canine plasma sample was spiked with standard peptides and again analyzed. The electropherogram obtained is shown in Fig. 7B. As can be seen by comparing Fig. 7A with Fig. 7B, only the Car and Ans peaks increased in size without other major changes in the electropherograms. Car and Ans contents in canine plasma were found to be in the range of 7.68 to 16.44 μM and 1.55 to 2.69 μM , respectively (Table 3). These results were in accordance with the data previously reported (i.e. $17.42 \pm 9.44 \mu\text{M}$ for Car, $1.89 \pm 0.92 \mu\text{M}$ for Ans) [23]. Hcar was not detected in canine plasma by the present method. To the best of our knowledge, there has been no report on the detection of Hcar in canine plasma. The precision of the analytical results was evaluated by repeatedly analyzing each canine plasma sample five times within a working day. The RSDs for the determination of Car and Ans were in the range of 2.6–4.1% and 3.2–4.8%. For the recovery studies, Car and Ans were added to the canine plasma sample and then analyzed. The recovery ranged from 93.1 to 105.5% (Table 3).

Conclusion

A MCE-CL method for the determination of carnosine-related peptides has been developed. The combination of pre-column CL labeling and post column use of a CL catalyst and enhancer allowed a highly sensitive detection of these peptides. Detection limits of the proposed MCE-CL assay were in the range of $2.8 \times 10^{-8} \sim 3.4 \times 10^{-8} \text{ M}$. The applicability of this assay to complex sample matrices was demonstrated. Quantification of trace carnosine peptides in human CSF and canine plasma samples could be accomplished within 2.5 min by using the present MCE-CL method. In the human CSF samples analyzed, Hcar was detected at the μM level, but Car and Ans were not detectable. In canine plasma, Car and Ans were detected, but not Hcar. The Car and Ans levels found in this work were in accordance with those reported in literature.

Abbreviations used

MCE, microchip electrophoresis; CL, chemiluminescence; ABEI, N-(4-aminobutyl)-N-ethylisoluminol; Car, carnosine; Hcar, homocarnosine; Ans, anserine.

Acknowledgments

Financial support from the National Natural Science Foundations of China (NSFC, Grant No. 20665002, 20875019 to SZ) and US National Institutes of Health (S06GM08047 to YML) is gratefully acknowledged.

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Fig. 1. Schematic diagram of the layout of the glass/PDMS microchip used in this work. S: sample reservoir; B: buffer reservoir; SW: sample waste reservoir; BW: buffer waste reservoir; R: the oxidizer reagent reservoir.

Fig. 2.

Effects of Co^{2+} concentration on CL intensity. The results were obtained by using a 15 mM borate running buffer (pH 9.8) containing 35 mM SDS, 1 mM adenine and Co^{2+} at different concentrations. The oxidizer solution was a 40 mM sodium bicarbonate buffer (pH 12.5) containing 90 mM H_2O_2 . Pinched injection for sampling by applying 750 V at S for 20 s, with SW held at ground, B at 240 V, BW at 320 V and R kept floating. The separation voltage was 2800 V, and the back voltage was 1700 V, with R at 550 V.

Fig. 3. Effects of adenine concentration on CL intensity. The results were obtained by using a 15 mM borate running buffer (pH 9.8) containing 35 mM SDS, 1 mM Co^{2+} and adenine at different concentrations. Other experimental conditions were as in Fig. 2.

Fig. 4. MCE-CL separation of ABEI-labeled peptides. Experimental conditions: MCE running buffer was a 15 mM borate running buffer (pH 9.8) containing 35 mM SDS, 1 mM Co^{2+} and 1 mM adenine; the oxidizer solution was a 40 mM sodium bicarbonate buffer (pH 12.5) containing 90 mM H_2O_2 . Other experimental conditions were as in Fig. 2. Peptide concentration was 8.0×10^{-7} M each.

Fig. 5.

Electropherogram obtained from the separation of a mixture containing DA, NE, GABA, Agm, Oct, Car, Hcar, Ans and 20 proteins amino acids. The concentration of each analyte is 1.0×10^{-6} M. MCE-CL conditions were as in Fig. 4. Peak identification: 1. ABEI; 2. Agm + Oct; 3. Arg; 4. Lys; 5. ABEI-DSC; 6. NE; 7. DA + Val; 8. Pro; 9. Asn; 10. Gln + Met; 11. Leu + Ile; 12. His; 13. Car; 14. Hcar; 15. Ans; 16. Phe; 17. GABA; 18. Ala + Trp; 19. Thr + Tyr; 20. Ser; 21. Cys; 22. Gly; 23. Glu; and 24. Asp.

Fig. 6. Electropherogram obtained from the separation of a human CSF sample (A) and the human CSF sample spiked with Hcar at 1.0×10^{-6} M (B). MCE-CL conditions were as in Fig. 4.

Fig. 7. Electropherogram obtained from the separation of a canine plasma sample (A) and the canine plasma sample spiked with Car and Ans at 1.5×10^{-6} M each (B). MCE-CL conditions were as in Fig. 4.

Table 1

Linearity (7-point) and detection limit of the proposed MCE-CL assay

Peptides	Linear regression equation ^{a)}	Linear range (10 ⁻⁸ M)	Correlation coefficient	Detection limit (S/N=3, 10 ⁻⁸ M)
Car	$H=4.897 \times 10^8 C - 2.568$	6.0–1500	0.9927	3.0
Hcar	$H=5.263 \times 10^8 C - 2.483$	6.0–1500	0.9975	2.8
Ans	$H=4.682 \times 10^8 C - 3.717$	6.0–1500	0.9991	3.4

^{a)}H, peak height (relative CL intensity, μV); and C, molar concentration of the analyte.

Table 2

Analytical results of Hcar in human CSF

Sample ^{a)}	Found (μM)	Added (μM)	Total found (μM)	Recovery (%)	RSD (% n=5)
CSFI	1.94	3.00	4.78	94.7	4.6
CSFII	2.73	3.00	5.86	104.3	3.8
CSFIII	4.25	3.00	7.03	92.8	3.3

^{a)} CSF samples were taken from patients with various neurological conditions.

Table 3

Analytical results of Car and Ans in canine plasma

Sample number	Peptide	Found (μM)	Added (μM)	Total found (μM)	Recovery (%)	RSD (%; n=5)
1	Car	10.46	20.00	31.50	105.2	2.9
	Ans	1.72	2.00	3.74	101.0	3.5
2	Car	7.68	20.00	26.30	93.1	4.1
	Ans	1.55	2.00	3.46	95.5	4.8
3	Car	16.44	20.00	36.78	101.7	2.6
	Ans	2.69	2.00	4.80	105.5	3.2