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Quantification of biogenic amines by microchip electrophoresis with chemiluminescence detection

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Abstract

A highly sensitive microchip electrophoresis (MCE) method with chemiluminescence (CL) detection was developed for the determination of biogenic amines including agmatine, epinephrine, dopamine, tyramine, and histamine in human urine samples. To achieve a high assay sensitivity, the targeted analytes were pre-column labeled by a CL tagging reagent, N-(4-aminobutyl)-N-ethylisoluminol (ABEI). ABEI-tagged biogenic amines after MCE separation reacted with hydrogen peroxide in the presence of horseradish peroxidase (HRP), producing CL emission. Since no CL reagent was added to the running buffer, the background of the CL detection was extremely low, resulting in a significant improvement in detection sensitivity. Detection limits (S/N = 3) were in the range from 5.9×10^{-8} to 7.7×10^{-8} M for the biogenic amines tested, which were at least 10 times lower than those of the MCE-CL methods previously reported. Separation of a urine sample on a 7-cm glass/poly (dimethylsiloxane) (PDMS) microchip channel was completed within 3 min. Analysis of human urine samples found that the levels of Agm, E and DA were in the ranges of 2.61×10^{-7} to 4.30×10^{-7} M, 0.81×10^{-7} to 1.12×10^{-7} M, and 8.76×10^{-7} to 11.21×10^{-7} M (n=4), respectively.

Keywords

Microchip electrophoresis; Chemiluminescence detection; N-(4-Aminobutyl)-N-ethyl-isoluminol; Biogenic amines; Human urine

1. Introduction

Biogenic amines are a group of low-molecular-mass organic bases occurring in living organisms. They are formed mainly by enzymatic decarboxylation of natural amino acids. Catecholamines, such as epinephrine (E), noradrenalin, and dopamine (DA), are important neurotransmitters in the nervous system. They act via dopaminergic and adrenergic receptors, and are involved in the regulation of responses to stress, psychomotor activity, emotional processes, learning, sleep, and memory [1–2]. Agmatine (Agm), also a neurotransmitter [3], is known to improve locomotor function and to reduce tissue damage following spinal cord injury [4]. However, at certain levels biogenic amines have been deemed to promote adverse effects on human health. For example, a variety of malignant cell propagations have been allied with the presence of increased biogenic amine levels. It has been suggested that high levels of certain polyamines in urine might serve as an indication of cancerous tumors [5]. For example, it was

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reported that urine levels of total polyamines (i.e. putrescine, spermidine and spermine) in healthy subjects human and cancer patients were 2.01 and 44.74 ($\mu\text{g}/\text{mg}$ creatinine), respectively [6]. Histamine, tyramine, and phenylethylamine are known to be associated with headaches, rushes, nausea, and hypertension [7,8]. Therefore, quantification of biogenic amines in biological samples has long been of importance.

A number of methods have been developed to determine biogenic amines in biological samples. The widely used methods include gas chromatography [9,10], thin layer chromatography [11,12], ion-pair liquid chromatography [13], and high-performance liquid chromatography (HPLC) [14–18]. Among them, HPLC-based methods are the most widely used. Capillary electrophoresis (CE) has been proved to be a powerful separation technique. Recently, CE has been also used successfully in the detection of biogenic amines [19–22].

In recent years, microfluidic devices have attracted considerable interest for their potential of miniaturization and integration of an entire chemical or biological analysis process on a single chip. Microchip electrophoresis (MCE), which can be regarded as a miniaturized version of classical CE, is one of the most successful applications of microfluidics in analytical chemistry. MCE offers the possibility to miniaturize traditional analytical instrumentation with the advantages of speed, automation and volumetric reduction of samples, reagents and waste. It has been applied for electrophoretic separations for a wide range of biochemical and chemical applications [23,24] since MCE was introduced by Manz's group [25]. Recently, MCE was successfully applied to separate and detect biogenic amines with laser induced fluorescence (LIF) detection [26–28]. Although high sensitivity can be obtained with LIF detection, it essentially necessitates a complex and costing detector. Chemiluminescence (CL) detection due to its simple instrumental set-up, high sensitivity, and wide linearity has become an advantageous detection scheme for MCE [29,30]. CL detection does not require a bulky light source. This makes it easy to integrate MCE with CL on a small chip. In addition, since the background signal is negligible the optical detector can be operated at its maximum sensitivity. Therefore, CL is unequally suited for in-line detection following MCE separations. MCE coupled with CL detection was successfully applied for quantifying amino acids [31,32], proteins [33,34], and metal ions [35].

In this paper, we describe a sensitive MCE-CL method for assaying biogenic amines. The method was based on pre-column CL labeling of analytes with N-(4-aminobutyl)-N-ethylisoluminol (ABEI) to enhance the sensitivity of CL detection. Pre-column CL labeling with ABEI has been used in CL detection following CE separations of arginine, glycine and biogenic amines [36,37]. In MCE, the microfluidic channels are normally smaller than those in CE, which affects negatively the detection sensitivity. Actually, the sensitivity of the MCE-CL methods reported previously was not impressive: the detection of limits were usually in the range from 10^{-6} to 10^{-7} M. Using the proposed pre-column CL labeling approach, the CL detection sensitivity can be further improved because no CL reagent was added to the running buffer, and therefore, the background of the CL detection was extremely low. Six biogenic amines including agmatine, epinephrine, dopamine, tyramine, histamine and noradrenaline were separated within 90 s. The feasibility of the present method was evaluated by quantifying biogenic amines in human urine samples.

2. Experimental Section

2.1. Chemicals and solutions

Agm, E, DA, tyramine, histamine, glutathione (GSH), taurine (Tau), Y-amino-n-butyric acid (GABA), and 20 protein amino acids were obtained from Sigma (St. Louis, MO, USA). N-(4-Aminobutyl)-N-ethylisoluminol was purchased from Fluka (Buchs, Switzerland). N,N'-Disuccinimidyl-carbonate (DSC) was purchased from Aldrich (Milwaukee, WI, USA).

Hydrogen peroxide was obtained from Taopu Chemicals (Shanghai, China), sodium dodecyl sulfate (SDS) was provided by Shanghai Reagents (Shanghai, China). Horseradish peroxidase (HRP) was purchased from Dongfeng Biochemicals (Shanghai, China). All other chemicals used in this work were of analytical grade. Water was purified by employing a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA), and used throughout the work. The electrophoretic buffer was 20 mM phosphate buffer (pH10.0, adjusted with 1M NaOH solution) containing 10 μ M HRP and 25 mM SDS. The oxidizer solution was 20 mM phosphate buffer (pH11.0, adjusted with 1M NaOH solution) containing 110 mM H₂O₂. Stock solutions of ABEI and DSC were prepared in methanol and acetonitrile, respectively. Stock solutions of biogenic amines were prepared in methanol and diluted with water as needed. All solutions were filtered through 0.22 μ m membrane filters before use.

2.2. Microfluidic-CL System

The glass 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. Use of the X-Y translational stage allowed viewing any point of the microchannel for introducing samples and collecting CL emission. 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 HV power supply, voltage divider, and 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 introducing samples and MCE separation. A valuable practical aspect of the inverted microscope setup was the possibility of visually checking all field-controlled operations on the device through the eyepiece. The inverted microscope was placed in a black box.

2.3. Microfluidic device

Schematic layout of the glass/PDMS microchip (95 mm×25 mm) is illustrated in Figure 1. The fabricated procedure of microchip was showed as following. First, the glass substrate with microchannels was fabricated through standard photolithography, wet chemical etching techniques [38]. Sylgard 184 PDMS prepolymer (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 $^{\circ}$ 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 the PDMS plate was exposed to the UV light (6W, mercury lamp) with the distance of 3 cm for 3 h, it joined with the glass substrate immediately, and the irreversible bonding was obtained. Microchannels measured 70 μ m wide by 25 μ m deep for sample introduction, separation and waste delivery, 250 μ m wide by 25 μ m deep for oxidizer introduction, respectively. All reservoirs were 4.0 mm in diameter and 1.5 mm deep (about 20 μ L capacity). 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.

2.4. Sample preparation

The human urine sample was obtained from healthy volunteers, which was stored at -20° C until analyzed. A 400 μ L urine sample in a 1.5 ml vial was added with double amount of acetonitrile and shaken vigorously for 5 min to precipitate proteins. After centrifuging at 16,000g for 10 min, the supernatant liquid was transferred into another 1.5 ml vial and dried

with a nitrogen stream. The residue was dissolved in 20 μL of 20 mM borate buffer at pH 9.0. The solution was vortexed and kept at 4 $^{\circ}\text{C}$.

2.5. Labeling

Labeling of biogenic amines with ABEI was carried out by following the procedure described by Kawasaki et al. [39]. First, a 25 μL of 5 mM ABEI solution was added to an equal volume of 5 mM DSC solution and allowed to react at room temperature for 2 h. This ABEI–DSC solution was then added to 10 μL sample solution. The mixture was allowed to stand at room temperature for 2 h. The labeled sample solution was diluted with running buffer prior to loading if necessary.

2.6. Procedures for separation and detection

The microchannels were rinsed with 1M NaOH for 30 min before the first use. Between two consecutive runs, the microchannels were rinsed sequentially with 0.1 M NaOH, water and electrophoretic buffer for 10 min each. First, the reservoirs B, S, SW and BW were filled with the electrophoretic buffer, reservoir R were filled with oxidizer solution and vacuum was applied to the reservoir BW in order to fill the separation channel with the electrophoresis buffer. Then, the electrophoretic buffer solution in reservoir S was replaced by sample solution. For loading the sample solution, a set of electrical potentials were applied to five the reservoirs: reservoir S at 800 V, reservoir B at 250 V, reservoir BW at 350 V, reservoir SW at grounded, and reservoir R floating. The sample solution was transported from reservoir S to SW in pinched mode. After 15 s, potentials were switched to reservoir B, S, SW and R at 2300, 1400, 1400 and 500 V, respectively, while reservoir BW was grounded for separation and detection.

3. Results and discussion

3.1. CL detection of ABEI labeled biogenic amines

In order to achieve a maximal detection sensitivity, effects of the oxidizer (i.e. H_2O_2) and catalyst (i.e. HRP) concentrations, and buffer pH on the CL intensity were investigated. In these experiments, the CL intensity (peak height) from assaying a mixture of biogenic amines (2.0×10^{-6} M each) was recorded. Experimental conditions were optimized based on the averaged results from three assays when the relative standard deviation (RSD) of each test point was less than 5.0%.

A concentration range of 50–130 mM was tested for H_2O_2 in the oxidizer solution. The results are shown in Figure 2. As can be seen, the CL emission increased with the increase in H_2O_2 concentration. However, the intensity of emitted light reached constant after the H_2O_2 concentration was greater than 100 mM. A 110 mM H_2O_2 solution was used for further studies. The reaction of ABEI labeled analytes with hydrogen peroxide was catalyzed by HRP. The CL intensity was significantly affected by the concentration of HRP in the running buffer. As shown in Figure 3, maximum CL emission occurred at 10 μM , and the CL intensity decreased sharply on either side of this concentration. It is not clear why CL signal decreased at higher HRP concentrations. However, it's worth noting that the presence of SDS micelles in the MCE running buffer might prevent free HRP molecules in the running buffer from being adsorbed onto the glass MCE channels. Oxidizer solutions of different pH values were also tested because the CL emission was pH dependent. It is well known that luminol (or isoluminol)- H_2O_2 CL increases with the increase in solution pH. However, considering the fact that H_2O_2 decomposes more quickly at high pH values, an oxidizer solution of pH 11.0 was selected for the post-column CL reaction.

3.2. MCE separation of biogenic amines

Our efforts to separate Agm, histamine, tyramine, E and DA by free zone MCE using a phosphate buffer went without success. Therefore, micellar MCE using SDS micelles was evaluated. The effects of SDS concentration were investigated in the range of 5 – 30 mM. The study showed that introducing SDS micelles into the system dramatically enhanced the separation. An optimal SDS concentration of 25 mM was selected for further studies. Electrophoretic buffers of various pH values were tested for the separation. In the pH range tested (9.0 – 11.0), the separation improved as increasing the buffer pH till pH = 10.0. Further increasing the buffer pH, the resolution between E and DA decreased significantly. A running buffer of pH 10.0 was selected for further studies.

3.3. Interference studies

ABEI, after coupling to DSC, reacts with primary and secondary amines, forming chemiluminescent ABEI derivatives [40]. Therefore, the influence of ABEI-amine derivatives on the quantification of the biogenic amines in biological samples was investigated. A mixture solution containing 20 protein amino acids, GABA, Tau, GSH and the five biogenic amines was prepared for this test. These compounds often occur in biological samples and most likely interfere with the quantification of biogenic amines. A typical electropherogram from separating the mixture is shown in Fig. 4. As can be seen, many compounds in this mixture were detected, producing many MCE-CL peaks. Fortunately, under the selected separation conditions no compounds co-eluted with Agm, histamine, tyramine, E and DA, which suggested that none of these compounds would interfere with the determination of the five biogenic amines.

3.4. Analytical figures of merit

The present MCE-CL method was evaluated in terms of the response linearity, limit of detection, and reproducibility. Six-point equally distributed calibration curves were prepared by assaying standard solutions at concentrations ranging from 2.0×10^{-7} to 4.0×10^{-4} M each amine (except Agm ranging from 1.5×10^{-7} to 4.0×10^{-4} M). Detection limits for the five amines were estimated from the calibration curves. The results are shown in Table 1. As can be seen, linear calibration curves were obtained with correlation coefficients (r^2) ranging from 0.9918 to 0.9975. The detection limits (S/N=3) were from 5.9×10^{-8} to 7.7×10^{-8} M. Assay reproducibility was studied by separating a standard solution (2.0×10^{-7} M each amine) for 7 times. The RSDs of peak height and migration time for all analytes were found < 4.6%.

3.5. Analysis of human urine samples

The present MCE-CL method was applied to the determination of trace biogenic amines in human urine samples. A typical electropherogram obtained from the analyses is shown in Fig. 5A. Three peaks corresponding to Agm, E and DA, respectively were well identified. To verify peak identification, the sample was spiked with these amines at 2.5×10^{-6} M each and separated again. The electropherogram obtained is shown in Fig. 5B. By comparing the two MCE traces shown in Fig. 5A and Fig. 5B, it can be seen that the peaks corresponding to Agm, E and DA increased in size without other major changes in the electropherogram. Table 2 summarizes the analytical results. Concentrations of Agm, E and DA in human urine were found to be in the range of 2.61×10^{-7} to 4.30×10^{-7} M, 0.81×10^{-7} to 1.12×10^{-7} M, and 8.76×10^{-7} to 11.21×10^{-7} M, respectively. These results were closely comparable with those obtained by HPLC methods [41,42] and CE-LIF methods [19]. Interestingly, histamine and tyramine were not detected in the samples analyzed. The basal levels of these biogenic amines in human urine may be too low to be detected by the present method. The assay precision was evaluated by repeatedly analyzing each human urine sample four times within a working day. RSDs for the three amines detected were between 2.4% and 4.7%. Recoveries of Agm, E and DA from this

sample matrix were also studied by adding authentic Agm, E and DA into urine samples, and the samples were then analyzed again. Recoveries were found to be in the range of 94.8 to 103.1%.

4. Conclusion

MCE with CL detection is known as one of the most interesting lab-on-a-chip devices. In this work, a new MCE-CL method for assaying biogenic amines was developed. To improve the assay sensitivity, pre-column CL labeling with ABEI was performed. Pre-assay labeling of the biogenic amines avoided adding the CL reagent into the system, which greatly diminished the CL background and hence the assay sensitivity. The applicability of the present method was demonstrated by analyzing human urine samples. Several important biogenic amines concluding Agm, E and DA in human urine were detected. The analytical results were in close accordance with those obtained by other methods based on HPLC or CE techniques.

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Fig. 1.

Layout and dimensions of the glass/PDMS hybrid microchip used in this work. S: sample reservoir; B: buffer reservoir containing 20 mM phosphate buffer (pH=10.0), 10 μ M HRP and 25 mM SDS; SW: sample waste reservoir; BW: buffer waste reservoir; R: oxidizer solution reservoir containing 20 mM phosphate buffer (pH 11.0) and 110 mM H_2O_2 .

Fig. 2.

Effects of H₂O₂ concentration on CL intensity. Electrophoretic electrolyte was 20 mM phosphate buffer (pH =10.0) containing 10 μM HRP and 25 mM SDS. The oxidizer solution was 20 mM phosphate buffer (pH 11.0) containing different concentration of H₂O₂. Potential applications for pinched sample injection and MCE separation were described in Experimental Section.

Fig. 3. Effects of HRP concentration on CL intensity. Electrophoretic electrolyte was 20 mM phosphate running buffer (pH =10.0) containing 25 mM SDS and varying concentrations of HRP. Other conditions were as in Fig. 2.

Fig. 4.

Electropherogram obtained from the separation of a mixture containing biogenic amines, GSH and amino acids. Electrophoretic electrolyte was 20 mM phosphate buffer (pH 10.0) containing 10 μ M HRP and 25 mM SDS. The oxidizer solution was 20 mM phosphate buffer (pH 11.0) containing 110 mM H_2O_2 . The pinched sample injection and separation operation voltage was showed in the procedure section for separation and detection. All analytes concentrations are 4.0×10^{-6} M. Peaks: 1. ABEI; 2. Agm; 3. Arg; 4. Lys; 5. ABEI-DSC; 6. histamine; 7. tyramine; 8. E; 9. DA; 10. Val; 11. Pro; 12. Asn; 13. Gln+Met; 14. Ser; 15. Leu+Ile; 16. Phe+Tau; 17. His; 18. Ala+GABA; 19. Thr+Trp; 20. Gly; 21. Tyr; 22. Cys; 23. GSH; 24. Glu; 25. Asp.

Fig. 5. Electropherograms obtained from the separation of a human urine sample (A) and the sample spiked with Agm, E and DA at 3.5×10^{-6} M each (B). The experimental conditions were as in Fig. 4. Peaks: 1. Agm; 2. E; 3. DA.

Table 1

Calibration curves and detection limits for the determination of five biogenic amines by the proposed MCE-CL assay

Analyte	Linearity ranges (10^{-7} M)	Regression equation ^a	R ²	LOD (10^{-8} M)
Agm	1.5–400	H=16.912δ 10 ⁷ C+2.023	0.9918	5.9
His	2.0–400	H=14.412δ 10 ⁷ C+2.903	0.9975	6.3
Tyr	2.0–400	H=11.168δ 10 ⁷ C+3.621	0.9943	7.5
E	2.0–400	H=10.234δ 10 ⁷ C+4.099	0.9969	7.7
DA	2.0–400	H=12.718δ 10 ⁷ C+2.575	0.9938	7.4

^aH: peak height (relative CL intensity, μV); C: concentration of analytes (M).

Table 2
Analytical results of agmatine, epinephrine, and dopamine in human urine

Sample	Found (10^{-7} M)			RSD (%; n= 5)			Added (10^{-7} M)			Total found (10^{-6} M)			Recovery(%)		
	Agm	E	DA	Agm	E	DA	Agm	E	DA	Agm	E	DA	Agm	E	DA
1	3.92	1.12	10.54	2.9	3.4	4.1	10.0	5.0	30.0	14.23	6.21	39.62	103.1	101.8	96.9
2	2.61	0.94	9.72	2.4	3.7	2.8	10.0	5.0	30.0	12.51	5.82	38.16	99.0	95.4	94.8
3	4.30	0.81	8.76	3.2	4.3	4.7	10.0	5.0	30.0	14.44	5.65	37.58	101.4	97.4	96.1
4	3.27	1.05	11.21	3.5	2.9	3.1	10.0	5.0	30.0	13.46	5.93	41.03	101.9	100.8	99.4