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Short communication

Indirect chemiluminescence detection for capillary zone electrophoresis of monoamines and catechol using luminol-$K_3[Fe(CN)_6]$ system

Yonggang Hu*, Xinxin Li, Zhitao Pang

College of Environmental Science and Engineering, Huazhong University of Science and Technology, Wuhan 430074, China

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Abstract

Indirect chemiluminescence (ICL) detection for capillary electrophoresis (CE) of monoamines and catechol using luminol-$K_3[Fe(CN)_6]$ system was described. A strong and stable background chemiluminescence (CL) signal can be generated by luminol-$K_3[Fe(CN)_6]$ reaction. Based on the principle of that some phenolic compounds may be oxidized in the presence of $K_3[Fe(CN)_6]$, quenching effect of catecholamines for luminol-$K_3[Fe(CN)_6]$ CL reaction results in a quantifiable decrease in the background signal. The conditions for CE separation and the CL detection for four standard catecholamines were systematically investigated using a homemade CE–ICL system. Under the optimum conditions, the detection limits of dopamine (DA), epinephrine (EP), norepinephrine (NE) and catechol (CA) were determined to be 0.18 μM 0.39 μM 0.48 μM and 0.09 μM, respectively. It also has been successfully applied to analyze seven pharmaceutical samples and seven human urine samples.

Keywords: Capillary electrophoresis; Chemiluminescence; Catecholamines; Urine sample

1. Introduction

Over the past 10 years, capillary electrophoresis (CE) has shown great promise in replacing many conventional clinical analysis methods, especially electrophoresis and high-performance liquid chromatography (HPLC) [1]. Biogenic amines, such as catecholamines and their metabolites have effects on nerve systems. Their determination from blood, urine and other body fluids is very important in the clinical diagnostics. Therefore, interest in testing the capability of CE combining different detection methods in screening and determining the catecholamines and their metabolites in urine samples has also increased recently [2–7]. However, prior to CE, purification treatment is usually necessary due to the complexity of the samples matrix. The pretreatment involves selectively extractive removal of the catecholamines from the urine matrix, e.g. by acid-washed alumina (Al_2O_3) at alkaline pH [2,6], hydrolysis [5] or polymer-based solid-phase extraction [3,7]. Obviously, a method requiring no sample work-up, such as clean-up steps would have advantages in diagnostic time, cost, labor, and having no analytes lost during the recovery steps.

Previous reports have shown that chemiluminescence (CL) is an alternative promising detection mode for CE separation [8–20]. Because few chemicals show the nature CL characteristic, derivatization of non-CL analytes prior to detection is generally required. However, derivatizating processes are often tedious and time-consuming. Furthermore, quantitative labeling of low-concentration analyte is also difficult. Alternatively, if the non-CL analytes suppress a CL reaction, then the analyte can be detected indirectly as an inverted peak where the CL intensity decreases from a normally high background. Tsai and Whang firstly investigated the feasibility of the use of CE–ICL method with luminol-H_2O_2–Cu^{2+} system for determination of catecholamines in "real life" sample [2]. However, as that point out by Tsai and Whang (1) application of the method mentioned above to human urine pretreated using the method described in [21], is still problematic due to the complexity of sample matrix

* Corresponding author. Tel.: +86 27 87544184 82; fax: +86 27 87792101.
E-mail address: yongganghu@163.com (Y. Hu).

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and the universal characteristic of the indirect chemiluminescence (ICL) method; (2) efforts on the development of the selectivity of CE–ICL or the appropriate sample preparation schemes are important and needed for complex biological samples analysis.

Recently, He and co-workers [22] and our group [23] has demonstrated that luminol-$K_3[Fe(CN)_6]$ CL system is favorable for CE–ICL detection because of less reagent variety, simple flow manifold, fast reaction velocity, high analysis sensitivity and strong inhibiting effect. Here, we develop a CE–ICL procedure for the determination of three monoamines and CA in pharmaceutical and urine samples using the luminol-$K_3[Fe(CN)_6]$ CL system. To our knowledge, for the first time, we demonstrated that the proposed method could be used for life samples analysis without complex pretreatment procedures as that described in [2,3,5–7], etc.

2. Experimental

2.1. Instruments

All CE experiments were performed on a homebuilt CE-CL detection system. The CE was driven by a high voltage power supply (Shanghai Institute of Atomic Nucleus, Shanghai, China) which provided a separation voltage up to +30 kV. The sizes of the capillaries (Yongnian Optical Fiber Factory, Hebei, China) for separation, post-column reaction and CL reagent delivering were 55 cm × 50 μm I.D., 15 cm × 530 μm I.D. and 25 cm × 200 μm I.D., respectively. The photons emitted from the CL reaction were detected by a photomultiplier tube (PMT) (Binsong, Peking, China). The PMT was operated at 843 V and photocurrent was magnified by a signal magnifier (Nanjing University, Nanjing, China) and then recorded using a PC computer controlled by data acquisition software (Qianpu Software Ltd., Shanghai, China).

The CL detection interface utilized an on-column coaxial flow design [9]. In brief, a 5 cm section of one end of the separation capillary was burned and etched with 0.1 mol/L HF for 2 h, and the etched section of the separation capillary was inserted into a reaction capillary in the middle of the 5 mm detection window. Then, the separation, reagent and reaction capillaries were jointed with a plexiglass tee connector which served as the reaction tee. The CL reagent was delivered by gravity through a reagent capillary and reached to the reaction capillary. Mixing of the reagents took place in a designated section of the reaction capillary, which was placed in front of the PMT with its protective polyimide coating removed. The end portion of the reaction capillary exited the detector and entered a buffer reservoir to complete the circuit. The ground in the system was the grounding electrode which was dipped in a buffer reservoir. The whole CL detection system was held in a light-tight box constructed from wood to avoid stray light.

2.2. Chemicals

The doubly distilled water was made with a doubly distilled water system (SZ-93, Shanghai yarong biochemistry instrument factory, Shanghai, China). DA, EP, NE were obtained from Sigma (USA); luminol was purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical reagent grade (China). All solutions were prepared in doubly distilled water and filtered through 0.22 μm pore-size membrane filter before being used. The pharmaceutical and human urine samples were obtained from the Huazhong University hospital (Wuhan, China). The urine samples were collected in a dark glass container, and analyzed within 2 h after collection.

2.3. Procedure

All new capillaries were sequentially rinsed using a syringe with 0.1 M NaOH, 0.1 M HCl, and doubly distilled water for 10 min, respectively. Then, the separation capillary was equilibrated 1 h with CE running buffer by pressure mode. The reagent capillary was initially filled with the $K_3[Fe(CN)_6]$ solution using a syringe. Electrophoretic separation was performed in 15 mM borate–2.0 mM luminol buffer (pH 9.5). During the analysis, the $K_3[Fe(CN)_6]$ solution reservoir was placed 20 cm above the outlet of reaction capillary. The sample solution was introduced at the positive electrode side for 10 s from a height of 20 cm by siphoning, and a voltage of 10 kV was applied for 2 min before each sample was injected.

3. Results and discussion

3.1. Optimization of ICL detection

In this study, CL reagents are normally introduced by post-column method to avoid the influence on CE separation. Luminol was added in the running buffer and introduced into the separation capillary by electroosmotic flow. $K_3[Fe(CN)_6]$ was delivered into the interface via gravity. The quantization of test catecholamines was carried out by measuring the net CL intensity $\Delta I = I_0 - I_i$, where I_0 is the CL intensity of baseline signal of luminol-$K_3[Fe(CN)_6]$ reaction and I_i, the signal caused by the analyte.

It was found that the CL intensity elevated as $K_3[Fe(CN)_6]$ concentration increased, and CL intensity reached to the maximum at 0.1 mM of $K_3[Fe(CN)_6]$. When the concentration of $K_3[Fe(CN)_6]$ was above 0.1 mM, the CL intensity became lower. The CL intensity decreased probably due to higher concentrations caused self-absorption of $K_3[Fe(CN)_6]$ as reported previously [24]. The effect of $K_3[Fe(CN)_6]$ pH variation on CL intensity in the CE–ICL system was also studied by means of different NaOH concentrations. We noticed that both noise and inhibiting signal increased with $K_3[Fe(CN)_6]$ alkalinity increased in the range 9.0–13.3. The best signal

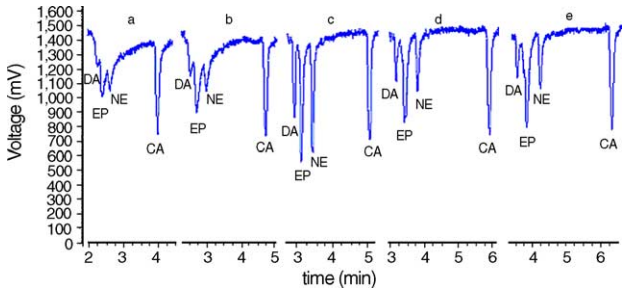

Fig. 1. Electropherogram of four analytes in different concentration of buffer. Separation and detection conditions: capillary, 55 cm × 50 μm I.D. fused silica; applied voltage, 20 kV; injection time, 10 s; running buffer, sodium borate −2 mM luminol; the post-column reagent, 0.1 mM potassium ferricyanide, pH 13.3; 1.3×10^{-5} MDA, 2.8×10^{-5} MEP, 2.4×10^{-5} MNE and 4.6×10^{-6} MCA. (a) 5 mM sodium borate −2 mM luminol; current, 10 μA. (b) 10 mM sodium borate −2 mM luminol; current, 17 μA. (c) 15 mM sodium borate −2 mM luminol; current, 23 μA. (d) 20 mM sodium borate −2 mM luminol; current, 32 μA. (e) 25 mM sodium borate −2 mM luminol; current, 42 μA.

to noise ratio was acquired when the pH of $K_3[Fe(CN)_6]$ reached to 13.3.

The influence of the luminol concentration on CL intensity in the CE–ICL system was examined within the range of 0.1–2.5 mM. At low concentrations, it was indicated that the CL signal of the four catecholamines and the baseline noise both significantly increased. The best signal-to-noise ratio was achieved when the concentration of luminol was 2.0 mM.

3.2. Optimum CE conditions

CE buffer composition, pH and concentration, are critical factors for CE–ICL analysis. Three kinds of buffer system, such as $Na_4B_2O_7$-luminol, $Na_4B_2O_7$–Na_2HPO_4-luminol and $Na_2B_2O_7$–Tris-luminol, were investigated, respectively. The best resolution and sensitivity for four test standard samples were obtained by the $Na_4B_2O_7$-luminol buffer system.

The influence of the buffer concentration on selectivity, resolution and ICL signals was investigated in our experiments. As shown in Fig. 1, $Na_4B_2O_7$ (15 mM)-luminol (2.0 mM) buffer system was evaluated to be the optimum buffer for CE–ICL analysis. The running buffer pH on the influence of separation was examined in the range of 9.0–10.0, it was demonstrated that the best pH value of running buffer for separation was 9.5.

For these test catecholamines, the voltage range studied was within the range of 15–25 kV. The best resolution and detection limits were obtained at the 20 kV separation voltage. The running buffer solution in two electrophoresis reservoirs was replaced after each separation to retain the stability of the chemiluminescence baseline, for the reason that the luminol was easily electrooxidized by CE's high voltage [22].

3.3. Dynamic range, limit of detection and RSD

Under the optimum conditions, the electropherogram shown in Fig. 1c indicated that four standard catecholamines were baseline separated in less than 6 min. The calibration curves were established using peak heights versus concentration levels. As shown in Table 1, the determination coefficients ($r^2 > 0.96$) show that good linearity can be obtained by this method. The concentration limits of detection (CLOD) for these analytes (S/N = 3) were also listed in Table 1 with range from 0.09 μM for CA to 0.48 μM for NE. Obviously, these CLODs are lower than that obtained with the Cu^{2+}/Co^{2+} catalyzed indirect system [2,19]. For 1.3×10^{-5} M and 5.7×10^{-7} M DA, 2.8×10^{-5} M and 9.2×10^{-7} M EP, 2.4×10^{-5} M and 8.5×10^{-7} M NE, 4.6×10^{-6} M and 1.2×10^{-7} M CA, the relative standard deviation (RSD) values on peak height were determined to be within the range of 2.1–4.5% by five replicated injections of each analyte.

3.4. Applications

This method was applied to the determination of monoamines in three clinical pharmaceutical preparations. The electropherograms of the three samples are shown in Fig. 2a–c. Because of their simple matrices, only a single inversion electrophoretic peak corresponding to the individual target analyte was observed in each sample. The results are summarized in Table 2 along with the labeled content of each analyte by four replicated injections. The measured contents of DA, EP and NE compare favorably with their labeled value in the respective sample, the *t*-test comparison of the means for the developed method and the labeled values

Table 1
Parameters of regression equations for the four analytes

Substance name	Linear range (M)	Regression equations	Determination coefficient (r^2)	Limit of detection (μM)			RSD (%) ($n = 5$)
Dopamine	2.6×10^{-7} to 5.3×10^{-5}	$Y = 2 \times 10^7 X + 251.2$	0.9698	0.18	2.2 [2]	3.6 [19]	2.9
Epinephrine	5.5×10^{-7} to 5.5×10^{-5}	$Y = 2 \times 10^7 X + 156.3$	0.9830	0.39	0.9 [2]	1.9 [19]	3.1
Norepinephrine	4.9×10^{-7} to 4.9×10^{-5}	$Y = 2 \times 10^7 X + 113.8$	0.9856	0.48	1.6 [2]	3.7 [19]	3.3
Catechol	9.1×10^{-8} to 9.1×10^{-6}	$Y = 1 \times 10^8 X + 219.6$	0.9775	0.09	0.5 [2]	7.5 [19]	2.1

Separation and detection conditions: capillary, 52 cm × 50 μm I.D. fused silica; applied voltage, 20 kV; current, 23 μA; injection time, 10 s; running buffer, 15 mM borate–2.0 mM luminol, pH 9.5; the post-column reagent, 0.1 mM potassium ferricyanide, pH 13.3. In [2], catecholamines were detected by luminol-H_2O_2–Cu(II) system. In [19], catecholamines were detected by luminol-H_2O_2–Co(II) system.

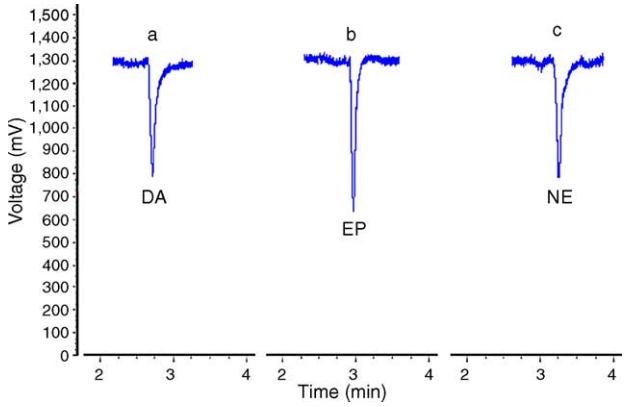

Fig. 2. Electropherogram of three pharmaceutical preparations in the optimized conditions. (a) dopamine hydrochloride injection diluted 10,000:1 with the electrophoretic buffer. (b) Epinephrine hydrochloride injection diluted 250:1 with the electrophoretic buffer. (c) Norepinephrine bitartrate injection diluted 500:1 with the electrophoretic buffer.

Table 2
Results of pharmaceutical preparations analysis

Sample	Labeled (mg/mL)	Measured[a] (mg/mL)
Dopamine hydrochloride injection	20	20.32 ± 0.59
Epinephrine hydrochloride injection	1	0.98 ± 0.03
Norepinephrine bitartrate injection	2	1.96 ± 0.06

The conditions of CE and reaction were as same as in Table 1.
[a] Data are represented as mean ± SD ($n = 4$).

revealed that there is no evidence of significant differences at the 95% confidence level.

As can be seen in Fig. 3, a urine sample was subjected to analysis by this developed method. Apparently, the interference on the baseline signal caused by complex matrix, which severely interfere in the UV [4,5] and fluorescence detection [6], was well-resolved in our experiments. The peak size of the signal marked "DA" in Fig. 3B increased obviously with a standard addition. In this situation, three urine samples were

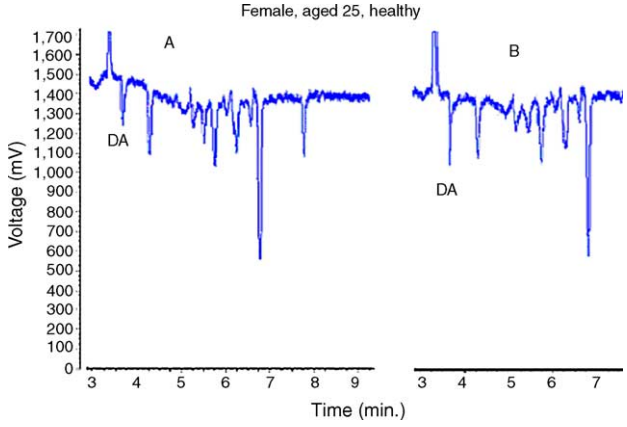

Fig. 3. Electropherogram of the urine sample 1 (five-flod dilution) in the optimized conditions. (A) Human urine sample and (B) urine sample spiked with 2.7×10^{-6} M DA.

Table 3
Determination results of three urine samples

Urines	Content (M)	Regression equation[a]	Determination coefficient (r^2)
Sample 5	4.8×10^{-6}	$Y = 1 \times 10^8 X + 92.5$	0.9976
Sample 6	2.9×10^{-6}	$Y = 2 \times 10^8 X + 3.5$	0.9969
Sample 7	4.1×10^{-6}	$Y = 1 \times 10^8 X + 95.3$	0.9952

[a] Where Y is peak height, x is the concentration of DA (M).

separated, and standard addition method was used to determine the DA quantitatively with standard addition calibration curve for each sample. The linear regression equation, determination coefficient and determination results were listed in Table 3.

4. Conclusions

It is clear that the proposed is useful for the analysis of DA, EP, NE and CA in pharmaceutical samples. In addition, for urine, DA can be determined with a simple treatment of sample. The EP, NE and CA signals did not found in the urine, it may be due to catecholamines easily oxidized with no addition of antioxidants (such as ascorbinic acid and EDTA) in our collection procedure. Therefore, the collection procedure should be improved in future study.

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