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On-line chemiluminescence determination protocatechuic aldehyde and protocatechuic acid in pharmaceutical preparations by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) with on-line direct chemiluminescence (CL) detection was first used in detecting protocatechuic aldehyde (PAH) and protocatechuic acid (PA) in their pharmaceutical preparations. It was found that the weak CL produced from the reaction of luminol with ferricyanide in an alkaline solution was strongly increased by PAH and PA which was separated by CE. Parameters affecting separation process and CL detection have been examined in detail. Under the optimum conditions, the baseline separation of PAH and PA was obtained within 6 min. The relative standard deviation (R.S.D.) for the analysis of PAH and PA was less than 1.1% for the migration time and 1.6% for the peak height. The detection limits ($S/N = 3$) of PAH and PA were 7.0×10^{-8} M and 5.0×10^{-8} M, respectively. The proposed method has been satisfactorily applied to the determination of PAH and PA in Salivia miltorrhrza pharmaceutical preparations.

Keywords: Capillary electrophoresis; Chemiluminescence detection; Protocatechuic aldehyde; Protocatechuic acid

1. Introduction

Capillary electrophoresis (CE) has been evolved into a large family of high-resolution separation techniques over the past two decades. CE can surpass high performance liquid chromatography (HPLC) in terms of simplicity, resolution and economy [1]. The extremely small inner diameter of the capillary results in typical advantages, such as the ability to use small volumes of sample and reagents, low mass detection limits. Most commercially available CE equipment uses UV–vis detection because of its simplicity and flexibility. However, the use of UV–vis detectors results in poor concentration–sensitivity which remains a serious hindrance to the development of CE [2]. Electrochemical and laser-induced fluorescence (LIF) detectors are more sensitive, but not as flexible as the popular UV–vis detector. On the other hand, mass spectrometry (MS) detector is universal but expensive and requires complex instrumentation. Finally, Raman and nuclear magnetic resonance detectors have also been used with CE, but their concentration detection limits are no better than those of the UV–vis detector [3].

The development of techniques for improved detection has been a priority in CE research in the quest for enhanced sensitivity. An alternative approach to improving existing detection methodologies is the use of chemiluminescence (CL) reaction. CL is an effective detection system for CE because of its high sensitivity and selectivity [4–11]. This detection system uses a simple, inexpensive optical system that requires no light source, which avoids stray light and source instability problems, and provides low background noise and excellent sensitivity, comparable to that of LIF. As a result, various CL-based systems, including luminol, peroxyoxalate, acridinium

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esters and firefly luciferase, have been developed for ultrasensitive detection in CE.

Traditional Chinese medicine has been extensively used to prevent and cure human diseases for over a millennium in Oriental countries. Because of its low toxicity and good therapeutical performance, traditional Chinese medicine has attracted considerable attention in many fields. Salivia miltorrhrza is a type of herbal drug. It contains fat-soluble tanshinones and water-soluble phenols. Pharmacological tests showed that the water-soluble phenols, protocatechuic aldehyde (3,4-dihydroxybenzaldehyde) (PAH) and protocatechuic acid (3,4-dihydroxybenzoic acid) (PA) were the most effective in treating anticardium colic, improving flow in coronary arteries and inhibiting the aggregation of platelets caused by adenosine diphosphate [12]. So far, there were several literatures [13–16] reported the simultaneous determination of PAH and PA in pharmaceutical preparations such as TLC [13], RP–HPLC [14] and CE with UV [15] or amperometric detection [16], respectively. However, these methods were limited because of complex preparation procedures, high expenditure and low sensitivity. To our knowledge, simultaneous determination of PAH and PA with CE–CL method has not been reported. Therefore, in order to ensure the clinical effects, it is important to develop a simple and rapid method for monitoring the quality of PHA and PA of preparations of Salivia miltorrhrza.

This paper first reported the potential application of CE–CL technique to the direct detection of PAH and PA. A constant CL background was obtained by a steady flow of the reaction of luminol with ferricyanide in an alkaline solution. After the analytes were separated by CE, they entered the reaction zone and increased the CL intensity of luminol with ferricyanide which was the oxidant as well as catalyst [17–20] in alkaline solution, resulting in a positive peak. The analyte concentrations can be detected from the area of peaks.

2. Experimental

2.1. Apparatus

The CE–CL apparatus used in this study was similar to that described by Cheng and coworkers [21] with some modifications. A schematic diagram of the apparatus was shown in Fig. 1. A 0–30 kV power supply (Institute of Atom Nucleus, Shanghai) provided the applied high voltage. Separation capillary of 50 μm i.d. × 375 μm o.d. was from Yongnian Optical Fiber Factory (Hebei, China). A 5 cm coating section of one end of the separation capillary was burned and then etched with hydrofluoric acid for 1.5 h to about 200 μm o.d. (before etch the tip of capillary was sealed by wax to avoid the inner wall was etched). The hydrofluoric acid treated end of the separation capillary was then inserted into a reaction capillary of 530 μm i.d. These two capillaries were held in a plexiglass four-way joint. The post-column reagents (the potassium ferricyanide in sodium hydroxide medium) were delivered by gravity through a reagent capillary of 320 μm i.d. The outlet of the reagent capillary was also led to the four-way joint. Plexiglass nuts and polyimide ferrules were used to fix the above mentioned three capillaries inside the four-way joint. The grounding electrode was also put into the joint to complete the CE electrical circuit. The outlet of the reaction capillary was 2 cm lower than the other end to make the solution flow out of the reaction capillary more easily and quickly. A 1 cm detection window was formed on the reaction capillary by burning off the polyimide coating. In order to collect the most intensive CL signal, the detection window was situated just in front of the photon-counting photomultiplier tube (PMT). The distance between the reaction capillary detection window and PMT was 3 mm. The high voltage of PMT was set at −650 V. The CL emission was collected with a BPCL ultra-weak luminescence analyzer (Institute of biophysics, Chinese Academy of Science, Beijing) and then recorded using a computer with BPCL software. The whole CL detection system was held in a large light-tight box to exclude stray light.

The CL emission spectra were examined by using a LS-50B luminometer (Perkin Elmer, USA).

2.2. Reagents and solutions

Unless otherwise stated, all chemicals used were of analytical-reagent grade. PAH and PA were purchased from the National Drugs Laboratory of China (Institute for Drugs and Biological Products Testing, Beijing, China). Luminol was obtained from Shanxi Normal University (Xi'an, China).

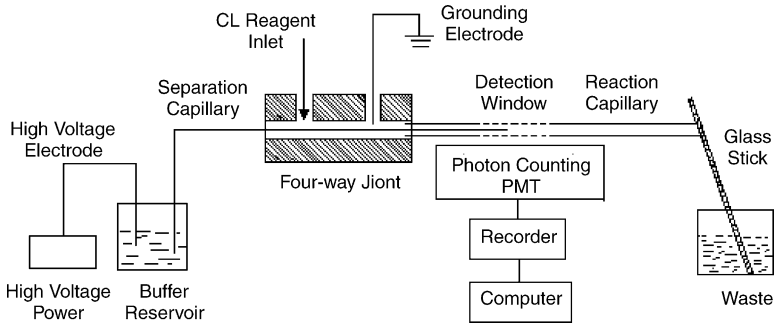

Fig. 1. Schematic diagram of the post-column CL detector for CE.

Potassium ferricyanide and sodium tetraborate were purchased from Shanghai Chemical Factory (Shanghai, China). Water purified with an Ultra-Pure Water System (Germany) was used to prepare all solutions. Stock solutions of PAH and PA were prepared in water, respectively. Others were prepared in water and diluted as needed. Salivia miltorrhrza tablets and Salivia miltorrhrza injection were purchased from a local market.

The running buffer containing 8.0 mM borate (pH 8.5) and 0.28 mM luminol was used in separating PAH and PA. The post-column CL reagent was the 1.25 mM potassium ferricyanide in 0.05 M NaOH medium. All solutions were filtered through a 0.22 μm membrane prior to use.

2.3. Procedure

It was very important to obtain reproducible and uniform inner walls of fused-silica capillaries so as to generate stable electro-osmotic flow (EOF) in capillary zone electrophoresis [22]. In this study, the new capillaries were rinsed sequentially with 2 M NaOH–CH_3OH (i.e. 2 g NaOH dissolved in 25 ml (4:1) methanol/water solution), 1 M NaOH, 1 M HCl and water for 30 min [23], and were then equilibrated with the running buffer solution for 30 min. The separation capillary was filled with running buffer while the four-way joint and reaction capillary were filled with post-column reagent. After each run, the separation capillary was treated with running buffer for 3–5 min. The sample was introduced by hydrodynamically for 10 s at a height difference of 10 cm. The applied voltage was 20 kV.

2.4. Sample preparation

Without any pretreatment, 2 ml injection sample was diluted to 100 ml with water and further diluted to the working range of the determination of PAH and PA, then analyzed according to the procedure described above.

The sugar-coats of Salivia miltorrhrza tablets was peeled off firstly. Five gram samples were carefully ground, and then a 1.5 g sample was accurately weighed and extracted with 10 ml methanol at room temperature for 24 h. The extracts were filtered through the same type of filter for three filtrations followed by centrifugation for 10 min at 5000 rpm. The supernatant was transferred to a 50 ml volumetric flask and adjusted to mark with water. This solution was further diluted with water appropriately so that the final concentration was within the working range.

Sample solution was filtered through a 0.22 μm membrane prior to injection.

3. Result and discussion

3.1. Effect of the running buffer pH

The pH of running buffer influenced electro-osmotic flow, resolution and sensitivity. Experiments were performed in the pH range of 8.0–9.5 and the results were shown in Fig. 2. When pH < 8.0, the response of PAH was low and the response of PA was difficult to measure. This was probably due to polyphenols becoming negatively charged and reactiving at basic pH. When pH ≥ 9.5, the CL intensity of the two analytes decreased and the peak of PA was relatively wide. And simultaneity current levels associated Joule heating increased markedly, which resulted in the decrease of sensitivity. Overall, running buffer at pH 8.5 was selected because of its similarity to the pH of borate buffer.

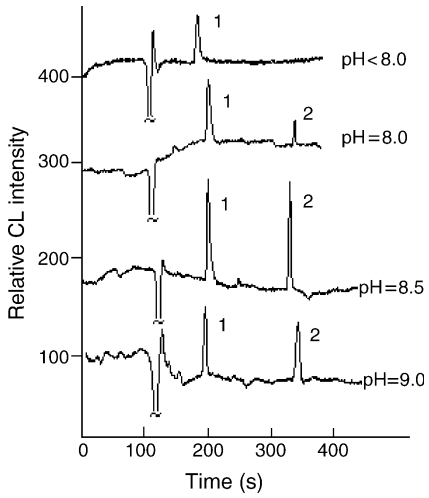

Fig. 2. Effect of running buffer pH on the CL intensity of PAH (1) and PA (2). Running buffer: 8.0 mM borate including 0.28 mM luminol; post-column CL reagent: 1.25 mM $K_3Fe(CN)_6$ in 0.05 M NaOH medium. Hydrodynamic injection for 10 s; applied voltage 20 kV; PAH 10 μM; PA 10 μM.

3.2. Effect of the running buffer concentration

The effects of borate running buffer concentration over the range 1–20 mM both on the migration time and the current were investigated. With the borate concentration varied from 1 to 20 mM, the migration time of PAH and PA were increasing slightly. However, with the borate concentration increasing, considerable Joule heating from the larger current would be also produced. When the concentration was lower than 4.0 mM, the peak of PAH became wide. For the sake of better resolution and shorter analysis time, 8.0 mM borate concentration was adopted for the method.

3.3. Effect of applied voltage

Changing the applied voltage from 12 to 24 kV showed that the applied voltage should be below 24 kV under the analytical conditions, in order to avoid excessive current inside the capillary. Therefore, 20 kV was chosen as the optimum applied voltage.

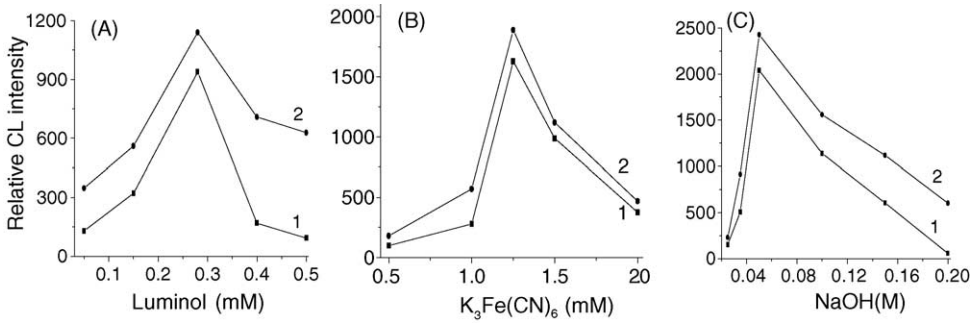

Fig. 3. Effect of the concentration of luminol (A), $K_3Fe(CN)_6$ (B) and NaOH (C) in post-column reagent on relative CL intensity. Other conditions as in Fig. 2.

3.4. Effect of introduction time of samples on CL intensity

The influence of the introduction time of samples was studied (5–30 s). Finally, an optimal introduction time of 10 s was selected since peak-height saturation occurred and peak-shape also broadened above this value.

3.5. Optimization of CL detection

Luminol concentration affected the CL intensity and sensitivity of PAH and PA detection. In this experiment, 0.05–0.5 mM luminol was tested. It was found (Fig. 3A) that increased CL intensity reached a maximum value when luminol concentration was 0.28 mM. Thus, the luminol concentration of 0.28 mM was chosen for subsequent research work.

CL intensity was found to be markedly affected by the concentration of $K_3Fe(CN)_6$. As shown in Fig. 3B, maximum light emission occurred at 1.25 mM $K_3Fe(CN)_6$, and the sensitivity decreased sharply on either side of this concentration. At below 1.25 mM $K_3Fe(CN)_6$, the CL intensity was proportional to the concentration of $K_3Fe(CN)_6$. At higher concentration of $K_3Fe(CN)_6$, the decrease of CL intensity was attributed to the deeper color of $K_3Fe(CN)_6$. Therefore, 1.25 mM $K_3Fe(CN)_6$ was adopted for the method.

In this study, the volume of the sample zone eluting from the 50 μm i.d. separation capillary was small enough compared to that of the reagent in the 530 μm i.d. reaction capillary, and hence the pH environment of the CL reaction was mainly dependent on the pH of reagent. Therefore, the effect of pH on CL intensity was investigated. The CL intensity was found to increase with increasing NaOH concentration, the maximum response was obtained when the concentration of NaOH was 0.05 M, as shown in Fig. 3C. When the concentration of NaOH was further raised, however, the CL intensity decreased.

The effect of NaOH concentration on the CL intensity was examined from 0.02 to 0.2 M (Fig. 3C). Maximum CL intensity was obtained when the concentration of NaOH was fed at 0.05 M. Thus, 0.05 M NaOH was chosen as optimum for further experiments.

3.6. Linearity, limit of detection, accuracy and precision

Under the above optimized conditions, PAH and PA were separated and determined. The calibration curves of PAH and PA were constructed (data not shown). They were constructed according to the following parameters: PAH, over a range of 0.60–30.0 μM with a detection limit of 7.0×10^{-8} M; PA, 0.20–10.0 μM with 5.0×10^{-8} M (3σ). The correlation coefficients were 0.999. The relative standard deviation (R.S.D.) of the migration time and the peak areas of each peak for three replicate injections were less than 1.1 and 1.6%.

The intra-day precision was determined within one day by analyzing three replicates control samples at concentrations of 0.4, 5.0, 35.0 μM for PAH and 0.1, 5.0, 13.0 μM for PA, respectively. The inter-day precision was determined on three separate days for the control samples. The intra-day and inter-day precision was defined as the R.S.D. and accuracy was determined by calculating the relative error (R.E.). The inter-day and intra-day accuracy and precision of the

Table 1
Intra-assay and inter-assay accuracy and precision for the determination PAH and PA

Analytes	Norminal concentration (μM)	Intra-day ($n = 3$) determined concentration (mean, μM)	Precision (% R.S.D.)	Accuracy (% R.E.)	Inter-day ($n = 3$) determined concentration (mean, μM)	Precision (% R.S.D.)	Accuracy (% R.E.)
PAH	0.400	0.415	1.32	3.75	0.41	3.25	2.50
	5.00	5.00	1.14	0.00	5.21	3.50	4.20
	35.0	34.2	0.990	−2.29	33.9	2.05	−3.14
PA	0.100	0.103	1.55	3.00	0.101	3.18	1.00
	5.00	5.05	1.39	1.00	5.00	2.12	0.00
	13.0	12.7	1.11	−2.31	13.5	2.54	3.85

Table 2
The results of Salivia miltorrhrza pharmaceutical preparations analysis ($n = 3$)

Samples of Salivia miltorrhrza	Compound	Diluted (μM)	Added (μM)	Total (μM)	R.S.D. (%)	Recovery (%)	Original amount
Tablet	PAH	2.41	2.40	4.69	2.30	95	0.57 (g/g)
	PA	0.86	0.60	1.45	2.00	98	0.42 (g/g)
Injection	PAH	1.13	1.10	2.25	1.80	102	14.8 (g/l)
	PA	0.99	0.60	1.58	1.60	98	7.69 (g/l)

analytical method were listed in Table 1. The R.S.D. of both intra-day and inter-day for PAH and PA were below 3.50%. The accuracy calculated as the relative error of intra-day and inter-day at low, medium, high concentrations were within the range of −3.14 to 4.20%.

3.7. Application

Salivia miltorrhrza tablets and injection prepared as above were analyzed by CE–CL. Fig. 4 showed the typical electropherograms of PAH and PA standard solution (a), Salivia miltorrhrza tablets (b) and injection (c), respectively. Peaks 1–3 have been confirmed to be PAH, PA and water by qualitative analysis, but there were some anomalous peaks in both Fig. 4b and c, which were probably interferences existing in Chinese pharmaceutical preparations of Salivia miltorrhrza, have not been identified. In order to examine the reliability of the method, the recoveries of PAH and PA in its pharmaceutical preparations were investigated by adding standard solutions of PAH and PA, and the results were shown in Table 2. The results demonstrated that the proposed method was highly sensitive, reproducible, and reliable for the determination of PAH and PA in its pharmaceutical preparations. It promised to be applicable to quantitative analysis of PAH and PA in Chinese pharmaceutical preparations of Salivia miltorrhrza.

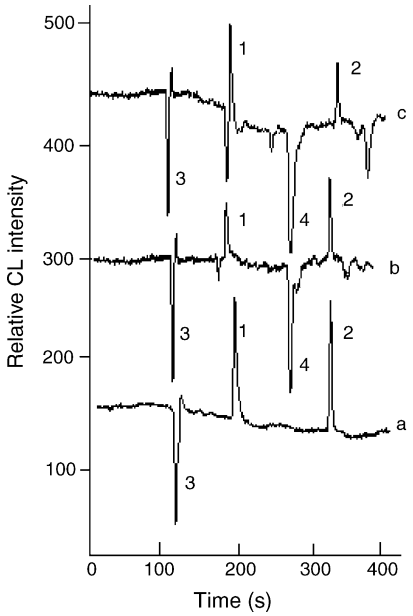

Fig. 4. The electropherogram of synthetic mixture of PAH and PA (a), Salivia miltorrhrza tablet (b) and Salivia miltorrhrza injection (c). Other conditions as in Fig. 2.

3.8. Possible mechanism of CL reaction

It was well known that the primary emitter is considered to be 3-aminophthalate (3-AP) [24–26] in the luminol CL reaction. In order to study the role of PAH or PA, we conducted some experiments in which reaction CL spectra were scanned with a LS-50B luminometer. The results showed only one peak at about 425 nm (same as the maximum emission spectra of 3-AP) with the absence and the presence of PAH or PA. This meant that the role of PAH or PA was only that of an enhancement reagent because there was no new emitter produced in the reaction.

Díaz and García [24] detailedly studied the enhanced CL mechanism of luminol CL induced by $K_3Fe(CN)_6$ enhanced by PA, and revealed that (i) the emitter was the luminol reaction product (3-AP) and not the enhancers; (ii) signals of enhanced reaction appeared at $K_3Fe(CN)_6$ concentrations higher than those of the enhancers; (iii) the reaction $K_3Fe(CN)_6$ with enhancers was faster than the reaction with luminol, and moreover, the protocatechuic radicals produced would not act on the luminol monoanion to convert it into a luminol radical; (iv) under alkaline conditions the reaction of the protocatechuic radicals with molecular oxygen took place and superoxide anion, the only reactive form of oxygen played an important role.

PAH and PA have analogous structure, the PAH enhanced mechanism was probably similar to that of PA with luminol–$K_3Fe(CN)_6$ system.

4. Conclusions

A method of on-line CL detection with CE separation of PAH and PA in Salivia miltorrhrza pharmaceutical preparations was described for the first time. Excellent separation efficiency was achieved using borate running buffer. The method was fast, simple and sensitive and indicated the feasibility of using the luminol–CL system for the analysis of the active components in complex Chinese traditional patent medicine. Further application of this system to the determination of other Chinese herb drug is in progress.

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