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Short communication

Determination of penicillamine in pharmaceuticals and human plasma by capillary electrophoresis with in-column fiber optics light-emitting diode induced fluorescence detection

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Abstract

In this paper, a capillary electrophoresis (CE) system with in-column fiber optics light-emitting diode (LED) induced fluorescence detection was developed for the determination of penicillamine (PA). The influence of buffer concentration, buffer pH, applied voltage and injection time was systematically investigated. Optimum separation conditions were obtained with 10 mM borate buffer at pH 9.1, applied voltage 20 kV and 8 s hydrodynamic injection at 30 mbar. The detection system displayed linear dynamic range from 3.2×10^{-7} to 4.8×10^{-5} mol L^{-1} with a correlation coefficient of 0.9991 and good repeatability (R.S.D. = 2.46%). The method was applied to the determination of PA in commercial tablets and human plasma, which the recoveries of standard PA added to tablets and human plasma sample were found to be in the range of 96.26–102.68 and 91.10–99.35%, respectively. The proposed method is cheap, rapid, easy, and accurate, and can be successfully applied to the formulation analysis and bioanalysis.

Keywords: Penicillamine; Capillary electrophoresis; Optical fiber; Light-emitting diode; Fluorescence detection

1. Introduction

Penicillamine (PA) is a strong chelating agent and can react with the majority of heavy metal ions. The outstanding metal-binding capability is reflected in the pharmaceutical importance of PA [1]. Thus PA is the drug of choice in the treatment of hepatolenticular degeneration (Wilson's disease) and is also effective for the treatment of several disorders including rheumatoid arthritis, primary biliary cirrhosis, scleroderma, fibrotic lung diseases, cystinuria, heavy element poisoning and progressive systemic sclerosis [2–5]. The typical dose administered to humans is 0.5–2.0 g daily. Whereas the D-enantiomer is therapeutic, the L-enantiomer is highly toxic since it possesses the same configuration as the L-amino acids (which are the constituents of proteins) and therefore interferes with the amino acid metabolism [6].

Several different methods have been proposed for the determination of PA in biological specimens and pharmaceutical formulations. Up to now, PA has been determined by HPLC with fluorescence [7], Chemiluminescence [8] and mercury-based electrochemical [9] detection, respectively. Various spectrophotometric methods [10–16] and electrochemical methods [17,18] also have been proposed for determination of PA. Recently, Torriero et al. have reported a method to determine PA using a micro-rotating biosensor [19,20]. Although these methods have been successfully employed, some of them suffer from interference from the pharmaceutical or biological matrix, and the others are time-consuming or require expensive equipment and consequently are not suitable for routine analysis in common laboratories. Therefore, a simple, rapid and sensitive determination of PA in pharmaceutical formulations and some biological specimens is of great importance.

Capillary electrophoresis (CE) is a powerful separation technique due to its minimal sample volume requirement, short analysis time, high separation efficiency and minimum operation cost [21,22]. It has been employed in the determination of a variety of compounds including pharmaceutical and biological

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samples. The versatility and number of ways that capillary electrophoresis can be employed suggest that almost all molecules can be separated using this powerful technique [23]. CE with UV [24] and LIF [25] detection has been applied for determination of PA in pharmaceutical and its metabolites in plasma, respectively. The major drawback of CE with UV detection is its relatively low sensitivity. CE with LIF detection is a sensitive analytical tool routinely used to monitor analytes of biological [26]. However, a number of drawbacks are associated with LIF detection. Primarily, conventional lasers are generally expensive and relatively bulky and have limited lifetimes [27].

The aim of the present work is to develop and validate a CE method suitable for the assay of the content of PA in pharmaceutical formulations and biological specimens. The proposed method employs a new CE system with in-column fiber optics LED induced fluorescence detector, which has been set up in our laboratory [28]. This detector uses a simple and inexpensive optical system, and avoids the light reflection and scattering on capillary surface and provides low background noise. The method has been constructed for the determination of PA tagged with naphthalene-2,3-dicarboxaldehyde (NDA), and successfully applied to the determination of PA in pharmaceutical formulations and human plasma.

2. Experimental

2.1. Chemicals and reagents

Naphthalene-2,3-dicarboxaldehyde (NDA) and D-penicillamine (D-PA, 3,3-dimethylcysteine) were obtained from Fluka (Buchs, Switzerland). Sodium borate and potassium cyanide were purchased from Sigma Chemicals (St. Louis, MO, USA). All the other chemicals and organic solvents used in this work were of analytical grade or above. Standard stock solution of PA (10 mM) was prepared in 0.10 M HCl solution and stored at 4 °C. It was further diluted as required concentration with 0.10 M HCl solution before use. NDA solution (2 mM) was prepared weekly in methanol and kept in dark at 4 °C. KCN solution (20 mM) and the borate buffer solution (pH 9.1; 10 mM) were prepared in water. Milli-Q water was used throughout the work. All solutions were filtered through a 0.45 μm membrane filter before being injected to the separation capillary.

2.2. Apparatus

High-voltage power supply (0–30 kV was obtained from Cailu (Beijing, China), capillaries were from Yongnian (Hebei, China), blue LEDs (3 mW) were from Shifeng Optic and Electronics Ltd. (Shenzheng, China) and all microscope objectives and filters were from Olympus (Japan). The fluorescence signal was subsequently detected by a PMT (CR105, Beijing Hamamatsu, China). The output signal was recorded and processed with a personal computer using in-house written software. The detail design of the in-column fiber optics LED induced fluorescence detection system for CE has been previously described [29].

2.3. Samples preparation

Twenty tablets (PA: 125 mg/tablet) were finely powdered and the equivalent of one tablet (125 mg as PA) was accurately weighed and extracted with 100 mL of 0.10 M HCl. It was sonicated at for 15 min with vortex mixing at 5 min intervals. The solution was filtered through an ordinary filter paper, washed with 0.10 M HCl solution several times and the filtrate plus washings were diluted to a 250 mL calibrated flask. The sample solutions were diluted with 0.10 M HCl solution to obtain solutions where the expected concentration of PA was within the calibration range before assay.

The human blood samples were obtained from a rheumatoid arthritis patient treated with oral doses of 1.2 g/day of PA and immediately centrifuged at 6000 × *g* for 15 min. The plasma obtained was transferred to 5-mL tubes and stored at −20 °C until analysis. A 0.50 mL of plasma sample was diluted with 0.50 mL of 0.60 M trichloroacetic acid solution and shaken vigorously for 1 min to deposit proteins, then left at 0 °C for 1 h. After being centrifuged at 10,000 × *g* for 20 min, the supernatant was transferred into another 1.5 mL vial and kept at 4 °C.

2.4. Precolumn derivatization

Ten microlitres of PA standard and treated sample solution was transferred, respectively, into 0.5-mL tube containing 140 μL of borate buffer solution. Fifty microlitres of 2.0 mM NDA and 50-μL of 20 mM KCN solution were added to the tubes, respectively. The solution was vortexed and kept at room temperature for 240 min. Then, the derivative sample solutions were injected for separation.

2.5. CE procedure

A new capillary was preconditioned by flushing with 1 M NaOH for 30 min before the first use. Between two consecutive injections, the capillary was rinsed sequentially with 0.1 M NaOH, water and running buffer for 3 min each. Samples solutions were injected into the capillary by hydrodynamic injection for 8 s at 30 mbar. After the introduction of derivative sample solution, 20-kV voltage was applied across the capillary. All separations were performed on a 55.0 cm × 100 μm i.d. (365 μm o.d. and 53.0 cm effective length) uncoated fused-silica capillary with borate buffer (pH 9.1; 10 mM). Fluorescence was excited by a blue LED with a maximum wavelength at 445 nm and the emission from the sample solution was detected after passing through a spatial filter and a yellow color filter (510 nm cut-on). All CE procedures were conducted at 25 °C.

3. Results and discussion

3.1. Method optimization

3.1.1. Choice of LED

Fig. 1 shows the excitation and emission spectra of NDA-labeled PA as well as the emission spectrum of LED used in this work. As can be seen, NDA-labeled PA is highly fluores-

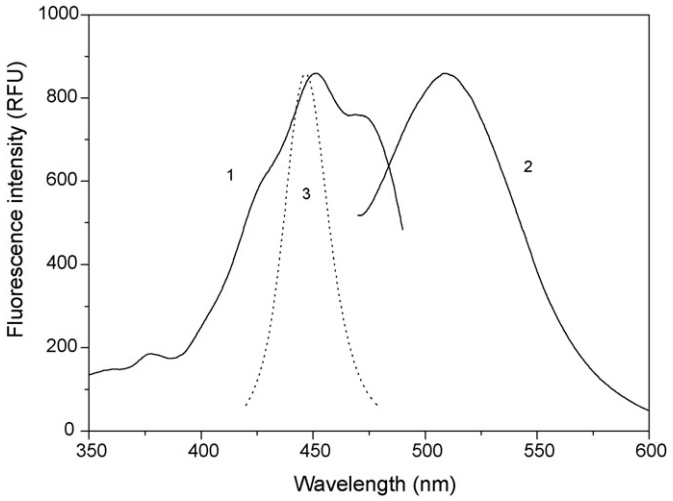

Fig. 1. Typical excitation (1) and fluorescence spectra (2) of NDA-labeled PA in running buffer. The dashed line (3) shows the emission spectrum of LED.

cent with an excitation maximum at 452 nm and an emission maximum at 508 nm. Because the fluorescence compounds have only a small Stoke's shift ($\lambda_{em} - \lambda_{ex} = 56$ nm), if the maximum wavelength of LED approaches the fluorescence emission of the NDA-labeled PA, then their spectrum overlap phenomenon will be very serious. When a blue LED with a maximum wavelength at 445 nm was used, there is only slight overlapping with the fluorescence emission of NDA-labeled PA. Although the excitation efficiency at 445 nm is lower than that at 452 nm in some sort, higher detection sensitivity can still be obtained because of low background and noise [30]. So a LED with a maximum wavelength at 445 nm was chosen for this experiment.

3.1.2. Derivatization conditions

Fig. 2 shows the effect of the derivatization reaction time on the fluorescence intensity within 360 min. It was found that the derivatization reaction has gone to completion and the largest fluorescence intensity was obtained at ca. 220 min in the dark, so 4 h was selected as the optimum derivatization reaction time. It was also found that the PA derivative was stable, and the largest peak height was still obtained after having sustained for 10 h at 4 °C in the dark.

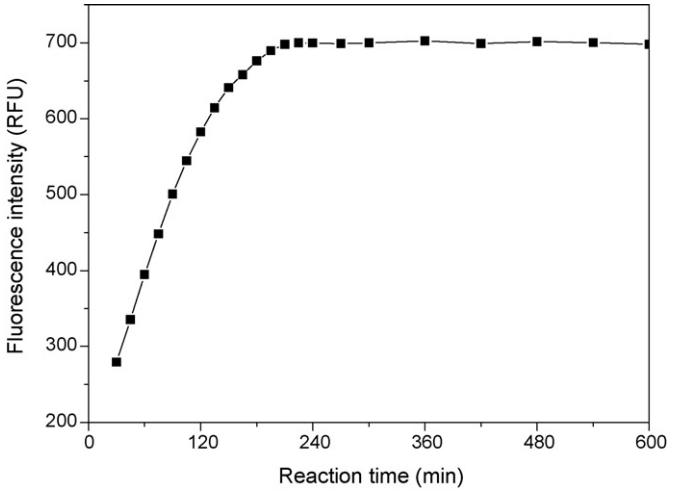

Fig. 2. Effect of derivatization reaction time on the fluorescence intensity of NDA-labeled PA.

3.1.3. Separation conditions

The effect of pH and concentration of the buffer was investigated. It was found that there was no significant difference among the migration time, fluorescence sensitivity and the resolution of NDA-labeled PA when the pH value of the running buffer solution changed from 8.0 to 10.0 and its concentration changed from 5 to 25 mM. Therefore, in considering the separation efficiency, migration time and peak symmetry, 10 mM borate buffer at pH 9.1 was chosen as the running buffer in this work.

When the applied voltage was varied in the range of 15–25 kV, the migration time of NDA-labeled PA was decreased with the applied voltage being increased as expected. It was decided to use an applied voltage of 20 kV in all subsequent work, because

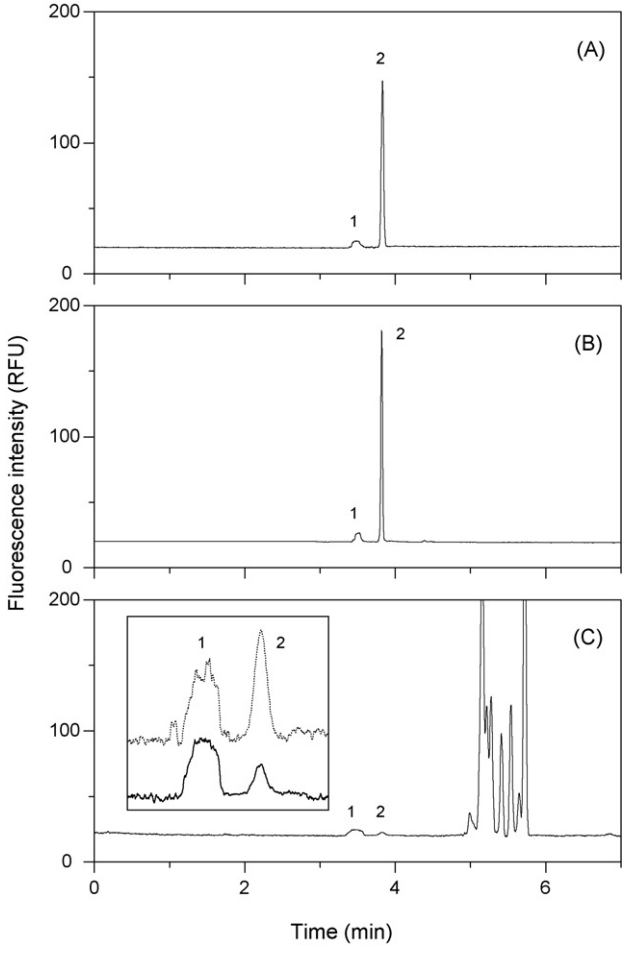

Fig. 3. (A) Electropherogram of a standard solution (8.0×10^{-6} mol L^{-1} for PA). (B) Typical electropherogram of a tablet sample solution. (C) Typical electropherogram of human plasma sample solution, where the dotted trace was obtained from sample spiked with 2.0×10^{-6} mol L^{-1} PA. Peak identification: (1) NDA and (2) NDA-labeled PA. Fused-silica capillary: 55.0 cm (53.0 cm to the detector window) × 100 μm i.d.; buffer: borate buffer (pH 9.1; 10 mM); applied voltage: 20 kV; hydrodynamic injection for 8 s at 30 mbar.

Table 1
Repeatability of peak area, peak normalization, ratio of peak area and ratio of peak normalization values for 4.0×10^{-6} mol L^{-1} NDA-labeled PA ($n = 10$)

Parameter	Migration time (min)	Peak area	PN
Mean ± S.D.	3.82 ± 0.02	4.86 ± 0.12	1.28 ± 0.04
R.S.D. (%)	0.52	2.46	3.12

PN (peak normalization), peak area/migration time; S.D., standard deviation; R.S.D., relative standard deviation.

high applied voltages can result in Joule heating that ruins the resolution and repeatability and the instrument employed did not have a thermostat facility.

Investigation was performed by changing injection time from 2 to 20 s at 30 mbar. When the injection time is more than 8 s, the peak widths of NDA-labeled PA were increased and the peak shapes were deformed. Therefore, 8 s was chosen as the optimum injection time.

Through above experiments, the optimum conditions for the determination of PA were chosen as follows: 10 mM borate buffer at pH 9.1, applied voltage 20 kV, hydrodynamic injection for 8 s at 30 mbar. The typical electropherogram of standard solution of NDA-labeled PA is shown in Fig. 3A.

3.2. Method validation

3.2.1. Linearity

To test the response linearity, series standard solutions of PA were determined. Typical calibration line (obtained using the least-squares method) could be expressed as $Y = 1.1577X – 0.2667$ ($r = 0.9991$), where Y is the peak area ratio and X is the concentration in 10^{-6} mol L^{-1}. The calibration curves exhibited an excellent linear behavior over the concentration range of from 3.2×10^{-7} to 4.8×10^{-5} mol L^{-1}. The limits of detection and quantification were calculated by measuring the noise in different blanks, and taking into account a factor of 3 and 10 for limit of detection (LOD) and limit of quantification (LOQ), respectively, and by using the signal obtained from standard solutions in order to convert to concentration units. The LOD was 9.5×10^{-8} mol L^{-1} and the LOQ was 3.2×10^{-7} mol L^{-1} for PA, respectively, these relatively high concentrations can be ascribed to the relatively weak intensity of the LED. Clearly, the use of a laser source enable a more sensitive assay to be developed, but the present system has adequate sensitivity for the routine determination of PA in pharmaceutical formulations and some biological specimens.

3.2.2. Precision

The assay was investigated with respect to repeatability and intermediate precision. The repeatability of the system (while keeping the operating conditions identical) was examined by injecting 4.0×10^{-6} mol L^{-1} NDA-labeled PA with 10 replicate injections and they were evaluated by considering migration time, peak area and ratio of peak normalization values of NDA-labeled PA. The precision values with their R.S.D. are shown in Table 1.

Three different concentrations of NDA-labeled PA (in the linear range) were analyzed in six independent series in the same day (intra-day precision) and six consecutive days (inter-day precision) within each series every sample was injected three times. The R.S.D. values of intra- and inter-day studies varied from 1.62 to 3.13% and 2.47 to 3.37%, respectively, which showed that the intermediate precision of the method was satisfactory (Table 2).

3.2.3. Accuracy

The accuracy of a method is expressed as the closeness of agreement between the found value and reference value. It is

Table 2
Precision and accuracy of the developed CE method for the analysis of PA ($n = 6$)

Concentration (10^{-7} mol L^{-1})	Intra-day			Inter-day		
	Mean ± S.D.	R.S.D. (%)	R.E. (%)	Mean ± S.D.	R.S.D. (%)	R.E. (%)
4	4.09 ± 0.11	2.69	2.25	4.15 ± 0.14	3.37	3.75
40	39.30 ± 1.23	3.13	−1.75	38.91 ± 1.08	2.78	−2.73
400	392.31 ± 6.36	1.62	−1.92	395.40 ± 9.77	2.47	−1.15

R.E., relative error. R.E. (%) = 100 × ((mean concentration − nominal concentration)/nominal concentration).

Table 3
Determination results of PA in commercial tablets sample solutions ($n = 6$)

Samples	Proposed method					Pharmacopoeia method[a] (mg/tablet)
	PA supplement ($\times 10^{-7}$ mol L^{-1})	Found: mean ± S.D. ($\times 10^{-7}$ mol L^{-1})	Recovery (%)	R.S.D. (%)	Content: mean ± S.D. (mg/tablet)	
Tablet 1	0 50	42.52 ± 0.57 91.68 ± 1.64	98.32	3.02	122.62 ± 1.66	122.17 ± 2.28
Tablet 2	0 50	43.82 ± 0.88 95.16 ± 1.17	102.68	2.67	125.56 ± 2.52	126.08 ± 3.25
Tablet 3	0 50	43.26 ± 1.05 91.39 ± 0.80	96.26	1.83	124.07 ± 3.01	123.70 ± 3.64

[a] Potentiometric titration method.

Table 4
PA determination in human plasma ($n = 6$)

Human plasma samples	Concentration ($\times 10^{-6}$ mol L^{-1})			Recovery (%)	R.S.D. (%)
	Initial	Added	Found: mean ± S.D.		
Plasma 1	8.72 ± 0.13	20	26.94 ± 0.65	91.10	2.12
Plasma 2	7.25 ± 0.20	20	27.12 ± 0.42	99.35	1.68
Plasma 3	7.62 ± 0.16	20	26.83 ± 0.38	96.05	3.37

determined by calculating the percentage relative error between the measured mean concentrations and added concentrations at the same concentration of NDA-labeled PA. The results obtained for intra and inter day accuracy were low of 2.25 and 3.75%, respectively (Table 2).

3.3. Application

After preparation and precolumn derivatization, the tablet and human plasma samples were measured by the CE system. Analysis was performed under optimum conditions and the typical electropherograms obtained from these samples analysis were shown in Fig. 3B and C. Each sample solution was analyzed six independent determinations and each series were injected three times. Table 3 shows the results of the determination of PA in commercial tablets, which compared favourably with those obtained by pharmacopoeia method [5] and the recovery test was satisfactory. The concentration of PA in human plasma and the recoveries test results are shown in Table 4. The recoveries obtained are always higher than about 91% and lower than 104%, while R.S.D. is always lower than 4%.

4. Conclusions

An in-column fiber optics LED induced fluorescence detection for CE was developed for the analysis of pharmaceuticals and biological samples. Quantitative measurements of PA in tablets and human plasma had been demonstrated. The present detector was a sensitive, accurate, simple and economic detection scheme, which can be easily set up in laboratory, since an inexpensive LED was used as excitation source, and a cheap optical fiber for guiding the excitation light was utilized. The home-built detection system also could be applied to the determination of other components such as amino acids (AAs) and proteins in pharmaceutical formulations and some biological specimens.

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