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Short communication

Pharmacokinetics of amoxicillin in human urine using online coupled capillary electrophoresis with electrogenerated chemiluminescence detection

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ABSTRACT

A novel and sensitive method for the determination of amoxicillin (AM) in human urine has been established using capillary electrophoresis (CE) coupled with electrochemiluminescence (ECL) detection, based on the ECL enhancement of Tris(2,2′-bipyridyl) ruthenium(II) with AM. The effects of several factors such as the detection potential, the concentration and the pH of phosphate buffer, the electrokinetic voltage and the injection time were investigated. Under the optimal conditions, the linear concentration of AM ranged from 1.0 ng/ml to 8.0 μg/ml (with a correlation coefficient of 0.9999). The limit of detection was 0.31 ng/ml. The mean recovery was 95.77% with relative standard deviations of no larger than 2.2%. This method is quick (the total run time within 6 min). This method has been successfully applied to a pharmacokinetic study in human urine after oral administration of AM.

1. Introduction

Amoxicillin (AM) is a β-lactam antibiotic that is used to treat certain infections caused by bacteria, such as pneumonia, bronchitis, venereal disease, and ear, lung, nose, urinary tract and skin infections. It is also used before some surgery or dental work to prevent infection [1,2]. The analytical methods reported for the quantification of AM in biological fluids are mainly based on high-performance liquid chromatography [3–6], spectrophotometry [7,8] and capillary electrophoresis [9]; detection methods include amperometry [10], fluorometry [4], mass spectrometry [11] and UV [12,13]. However, the previous methods suffer from disadvantages such as low sensitivity, consumption of large sample volumes, taking long time for assay, needing complex pretreatments, or the requirement for expensive instruments. Hence, a more sensitive, effective and simple method is necessary for the detection of AM.

Capillary electrophoresis (CE), with advantages such as small sample volume, high separation efficiency and short analysis time, is a useful tool for rapid detection of biochemical and pharmaceutical analytes [14]. Electrochemiluminescene (ECL) detection with $Ru(bpy)_3^{2+}$ is a viable alternative for CE detection. ECL has the advantages of simplicity, inexpensive instrumentation, low background noise, high sensitivity, good selectivity, and wide dynamic linear range [15]. ECL has been applied to a variety of fields such as biosensors, immunoassays, and flow injection analysis [16]. The combination of ECL with CE showed several advantages: high selectivity to the specific analyte, fast analysis speed, easy operation, low reagent consumption, etc. [17,18]. CE with ECL detection using $Ru(bpy)_3^{2+}$ has been studied since the mid-to-late 1990s for the determination of a variety of analytes which were listed in some reviews [19–26]. In this work, it was found that the weak ECL signal from the electrochemically oxidization of $Ru(bpy)_3^{2+}$ was greatly enhanced by AM. Based on this observation, CE with ECL detection was applied to the determination and the pharmacokinetic study in human urine after oral administration of AM.

2. Experimental

2.1. Reagents and apparatus

All chemicals and reagents were of analytical grade. Doubly distilled water (DDW) was used here. AM was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tris(2,2′-bipyridyl) ruthenium(II) chloride hexahydrate was obtained from Alfa Aesar (A Johnson Matthey Company, Ward Hill, MA, USA). Buffer system was Na_2HPO_4/NaH_2PO_4 (Hunan Reagent Company, Hunan, China). All solutions were prepared with DDW, stored in the refrigerator at 0–4 °C (the stored solution did not change within 2 weeks) and filtered through 0.45 μm membrane filters (Shanghai Xinya Purification Material Factory, Shanghai, China) prior to use.

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The CE–ECL detection system (Xi'an Remax Tech. Ltd., Xi'an, China) consisted of a high-voltage power supplier for separation and injection, a potential control system, a chemiluminescence detection system and data processor. The output ECL intensity was amplified and recorded using the MPI-B software. The chemiluminescence detection system was composed of a three-electrode system, with 200 μm diameter Pt disk as working electrode, a Pt wire as auxiliary electrode, and a Ag/AgCl electrode as reference electrode. The CE–ECL detection cell has been described in previous paper [27]. A fused-silica capillary (Yongnian Optical Fiber Co., Hebei, China) with 75 μm inner diameter and 40 cm length was used for electrophoresis separation.

2.2. Procedure for calibration

Approximately 350 μl of $Ru(bpy)_3^{2+}$ (5 mM) in phosphate buffer (50 mM, pH 7.5) was placed in the ECL detection cell for ECL measurement. The $Ru(bpy)_3^{2+}$-phosphate solution was replaced every 3 h during the experiments to avoid errors in ECL measurement due to possible changes in $Ru(bpy)_3^{2+}$ concentration. The capillary was filled with 0.1 M NaOH for 24 h before use and was subsequently flushed with DDW for 30 min using a syringe. Prior to each run, the capillary was flushed with DDW and the corresponding running buffer for about 5 min, respectively.

In all experiments, samples were introduced from the anodic end of the capillary by electrokinetic injection at 12 kV for 8 s, separated in the capillary at 10 kV. Detection potential was fixed at 1.20 V. The running buffer (pH 9.5) contained 12 mM phosphate. The potential of the photomutiplier tube (PMT) was operated at 800 V. After a stable baseline ECL signal was reached, electromigration injection was used for sample introduction, and the electropherogram was recorded.

2.3. Sample preparation

Fresh urinary samples were taken from a healthy student volunteer in the laboratory. The volunteer had abstained from any medications during the week preceding the study. After an overnight urine was discharged, the participant drank 100 ml of water and then collected urine. The urine collected before dosing was employed as a blank. The volunteer took two tablets of AM with 200 ml of water and then urine samples were collected every 30 min for the first 2 h, every 1 h between 2 and 4 h, and finally every 2 h until 6 h after start. The volume of each urine specimen was recorded. In addition, the participant was not allowed to consume any other foods in the morning, and 200 ml of water was drunk after collected urine sample each time.

To eliminate the influence of ionic strength in sample and obtain clear electrophoretic profile, a modified Rurak's extraction procedure was done before electrophoresis [28]. Urine samples (200 μl) or the spiked samples were pipetted into clean centrifugation tubes, after addition of 2 ml of ethylacetate, the mixture was made alkaline with 50 μl of 0.1 M NaOH, and then shaken for 30 s, after centrifugation (15,000 rpm, 1.0 min), the upper layer was transferred into a tube and evaporated to dryness under a stream of dry nitrogen at 80 °C. Next, the inner wall of the tube was rinsed with 200 μl of methanol to concentrate the sample and evaporated off. The dry residue dissolved in 200 μl water was measured.

3. Results and discussion

3.1. Cyclic voltammetry of $Ru(bpy)_3^{2+}$ and AM

The ECL intensity is dependent on the rate of the light-emitting chemical reaction which is in turn dependent on the potential applied to the electrode. Cyclic voltammograms (Fig. 1A) and the corresponding ECL intensity (Fig. 1B) were recorded. Under the cyclic voltammetric conditions, the rise of ECL intensity of AM solution and buffer solution (Fig. 1B) started at about 1.10 V and increased significantly with the increase of potential. The ECL intensity of AM was higher than the background noise caused by the direct oxidation of $Ru(bpy)_3^{2+}$ at the high applied potential of 1.20 V. These observations indicated that AM can react with the ruthenium species in the electrochemiluminescence process, and it can enhance the emitted light intensity. AM contains a lactam and amide. According to the earlier reports [29–31], AM can enhance ECL emission. The mechanism of $Ru(bpy)_3^{2+}$/AM systems ECL is as follows:

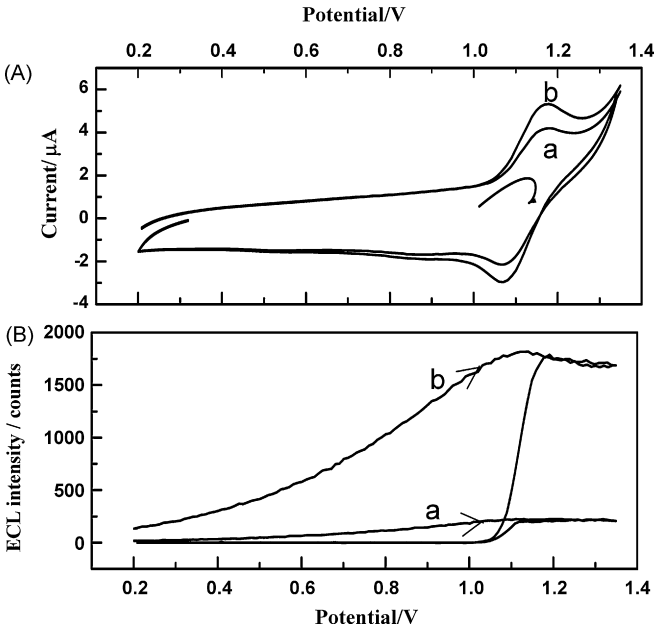

Fig. 1. Cyclic voltammograms (A) and profile of electrogenerated chemiluminescence (ECL) (B). Detection conditions: (a) 5.0 mM $Ru(bpy)_3^{2+}$ + 50 mM phosphate buffer (pH 8.0); scan rate, 100 mV/s; (b) 5.0 mM $Ru(bpy)_3^{2+}$ + 50 mM phosphate buffer (pH 8.0) + 1.0 μg/ml AM; scan rate, 100 mV/s.

$$Ru(bpy)_3^{2+} - e^- \rightarrow Ru(bpy)_3^{3+}$$

$$AM - e^- \rightarrow AM^{\bullet +} \rightarrow AM^{\bullet} + H^+$$

$$Ru(bpy)_3^{3+} + AM^{\bullet} \rightarrow Ru(bpy)_3^{2+\bullet} + \text{products}$$

$$Ru(bpy)_3^{2+\bullet} \rightarrow Ru(bpy)_3^{2+} + h\nu$$

3.2. Optimization of experimental conditions

3.2.1. Effect of the detection potential

In this work, the influence of applied potential on ECL intensity was investigated when using detection conditions of Fig. 1b. It was found that with the increase of the applied potential, the ECL intensity increased and reached a maximum value at 1.20 V, then decreased fast. Therefore, in the following experiment, the applied potential was set at 1.20 V.

3.2.2. Effect of the solution in ECL cell

According to early report, the ECL signal would vary with the concentration ratio of $Ru(bpy)_3^{2+}$ to the analyte [32]. A low concentration of $Ru(bpy)_3^{2+}$ leads to low background noise, while high

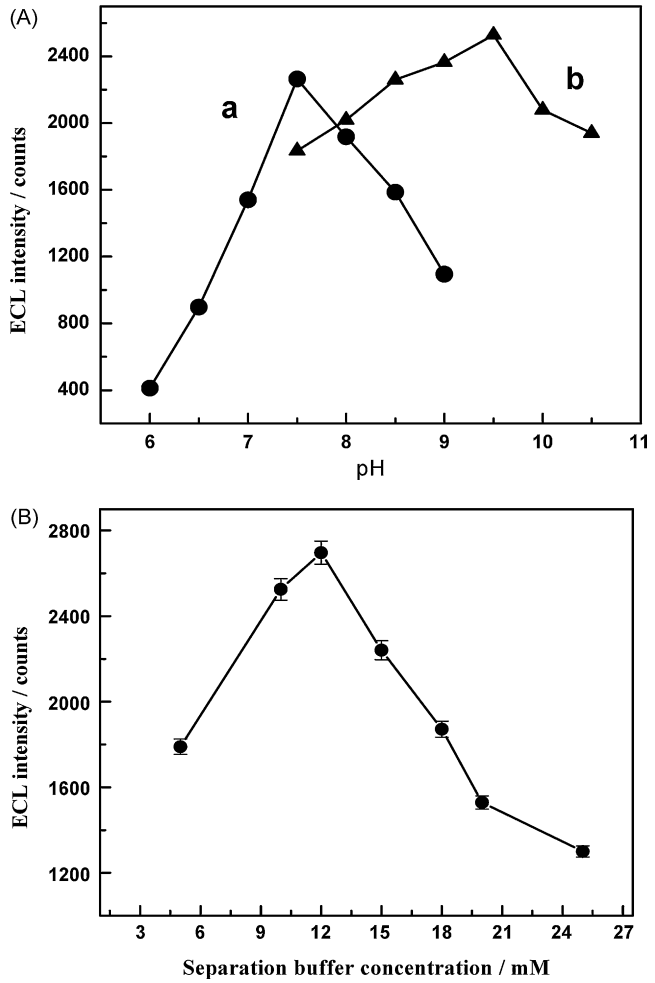

Fig. 2. Effect of phosphate buffer pH in ECL cell (A(a)) and separation buffer pH (A(b)) and separation buffer concentration (B) on ECL intensity. Detection conditions of A(a): sample, 1.0 μg/ml AM; detection potential, 1.20 V; electrokinetic injection, 12 kV × 8 s; separation buffer, 10 mM (pH 8.5) phosphate buffer; separation voltage, 10 kV; 5 mM $Ru(bpy)_3^{2+}$ and 50 mM phosphate buffer in ECL cell. Detection conditions of A(b): phosphate buffer pH 7.5 in ECL cell; other conditions as in A(a). Detection conditions of B: separation buffer, pH 9.5; other conditions as in A(a).

concentration leads to better sensitivity as well as the increase of S/N. But when the concentration is too high, more consumption of the expensive reagent $Ru(bpy)_3Cl_2{\cdot}6H_2O$ will be required and the working curve will not be linear any more. Therefore, in the experiment, 5 mM $Ru(bpy)_3^{2+}$ and 50 mM sodium phosphate were used as the optimized concentration in ECL cell.

The pH of detection buffer in ECL cell has a significant effect on ECL intensity of AM. As shown in Fig. 2A(a), it was investigated in a wide pH range of 6.0–9.0 in 0.5 pH units. When the pH value was 7.5, the ECL intensity reached the maximum value. ECL intensity then decreased with the increase of buffer pH. As a result, pH 7.5 was selected for all the following experiments.

3.2.3. Effect of separation buffer pH and concentration

The pH of the separation buffer plays an important role in CE separation and the ECL intensity. With the increasing pH of separation buffer from 7.5 to 10.5, the ECL intensity of AM increased and then decreased, the maximum ECL intensity occurred at pH 9.5 (Fig. 2A(b)). Therefore, the optimized pH value of separation buffer was 9.5.

The ECL intensity in the present work also changed as a function of the buffer concentration, which was plotted in Fig. 2B. With the increase of buffer concentration, ECL intensity ascends first and then descends with a turning point of 12 mM. In the following experiments, 12 mM phosphate buffer was used as the CE separation buffer.

3.2.4. Effect of separation voltage, injection voltage and injection time

The influence of separation voltage on the ECL intensity was carried out from 6 to 20 kV. ECL intensity increased with separation voltage increased up to 10 kV, and then it dropped as the voltage was further increased. The peak bottom width decreased rapidly when the separation voltage changed from 6 to 10 kV, but decreased slowly when the voltage was higher than 10 kV. Considering both of ECL intensity and peak bottom width, 10 kV was chosen as the separation voltage.

As a key factor in CE, the effects of injection time and injection voltage were studied in detail. The influences of injection voltage of 4, 6, 8, 10, 12, 14, and 16 kV on the ECL intensity were carried out. At higher injection voltage, more analyte can enter into the diffusion layer, so higher ECL signal may be produced. However, the analyte cannot reach the electrode surface immediately and diffuse into the solution, so the peak is retarded and broadened. So as a compromise of the high ECL intensity and the improved column efficiency, the injection voltage of 12 kV was recommended.

The injection time also affected the detection. ECL intensity was influenced by the injection time ranging from 4 to 16 s. When injection time changed from 4 to 8 s, the ECL signal increased proportionally. Beyond 8 s, the ECL intensity increased slowly. Furthermore, the longer injection time broadened the peak shape as a result of an excessively large injection volume. Therefore, 8 s was selected as optimum injection time.

3.3. Linearity, detection limit and reproducibility of AM

Determined a series of the AM standard solutions of different concentrations which contained 200 μl of blank urine sample. All urine samples were extracted as shown in Section 2.3 and examined with CE–ECL system. In order to correct the errors of injection volumes, the interstandard (ISTD) method of calibration was used in the determination of AM. Triethylamine (TEA) was selected as an ISTD compound.

Under the optimized experimental conditions: 1.2 V (versus Ag/AgCl) applied potential, 5 mM $Ru(bpy)_3^{2+}$ and 50 mM phosphate buffer (pH 7.5) in the detection cell, 12 mM running buffer (pH 9.5), 10 kV separation voltage, 12 kV injection voltage and 8 s injection time. The linear range and the detection limit were studied. Calibration curve for AM was linear over the concentra-

Table 1
Analytical results and recoveries of AM in human urine samples ($n = 5$).

Uptake urine time (h)	Determined (μg/ml)	Added (μg/ml)	Recovered (μg/ml)	Recovery (%)	R.S.D. (%)
1.0	0.3246	0.3000	0.2875	95.83	2.1
		0.5000	0.7850	92.08	1.9
1.5	0.4704	0.4000	0.3862	96.55	2.2
		1.0000	1.4566	98.62	2.0

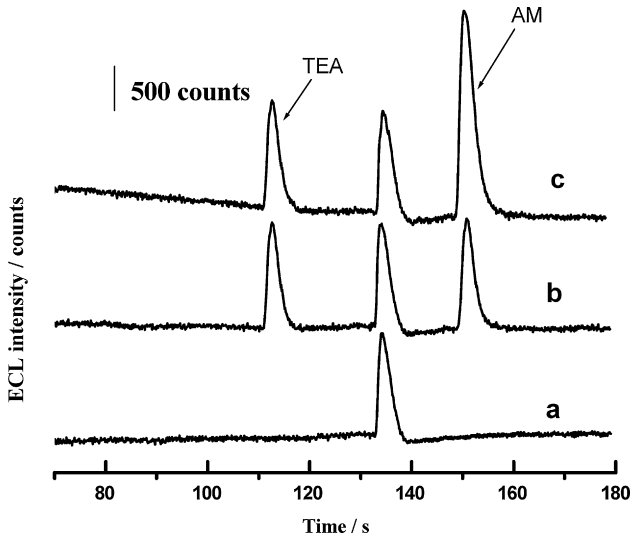

Fig. 3. CE–ECL electropherograms of (a) for blank urine sample, (b) for the human urine sample after taking an administered dose of AM for 1 h spiked with 1.0 μg/ml TEA and (c) for (b) spiked with 0.5 μg/ml AM; detection conditions: separation voltage, 10 kV; injection voltage, 12 kV; injection time, 8 s; other conditions as in Fig. 2B.

tion range from 1.0 ng/ml to 8.0 μg/ml with a regression curve of $y = 2526.4x + 210.12$ (y for peak height, x unit for μg/ml, $r = 0.9999$). The detection limit was 0.31 ng/ml with a signal-to-noise ratio of 3. The relative standard deviations (R.S.D.) of the ECL intensity (peak height) and the migration time for six consecutive injections of 1.0 μg/ml AM were 1.7% and 0.72%, respectively. The calibration was repeated every day within 5 days. The R.S.D. of the slopes and intercepts for 5 calibrations was less than 2.5%.

3.4. Application

To examine the application for practical analysis, the CE–ECL method was applied to the determination of AM and studied its pharmacokinetics in human urine. To determine the recovery of this method, the urine samples spiked with different concentrations of AM were detected. The recoveries were calculated by detecting the peak height of AM from the human urine sample spiked with known concentration of AM to that of AM added to the blank urine. The recoveries of AM in urine samples were 92.08–98.62% (Table 1). The R.S.D. of ECL peak intensity was 1.9–2.2%. The typical electropherogram was shown in Fig. 3. The peaks were identified by comparison the migration times and by spiking the standards to the sample solution. The peak of Fig. 3c at 150 s obviously increased than that of Fig. 3b. Therefore, the peak at 150 s was considered as the peak of AM. In order to test the interferences of other beta-lactam antibiotics on the determination of AM, 1 μg/ml of erythromycin, josamycin and norfloxacin each mixed with 1 μg/ml AM. They did not interfere the determination of AM.

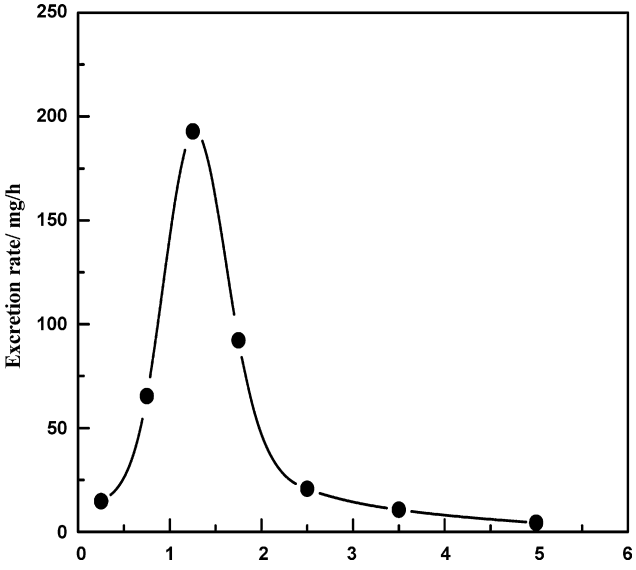

Fig. 4. Excretion rate of AM in the urine. Detection conditions: 1.2 V (versus Ag/AgCl) applied potential, 5 mM $Ru(bpy)_3^{2+}$ and 50 mM phosphate buffer (pH 7.5) in the detection cell, 12 mM running buffer (pH 9.5), 10 kV separation voltage, 12 kV injection voltage and 8 s injection time.

3.5. Pharmacokinetics of AM in human urine

The concentrations of AM in the urine (substituted by "C") were determined by the proposed CE–ECL method. The medicine discharge capacity of this segment (substituted by "X") is defined as urine volumes multiply C. The average discharge rate of AM in the urine (substituted by "$\Delta X/\Delta t$") is defined as difference of X divided interval of dwell times (substituted by Δt), The relation of average excretion rate of AM in the urine (educed by $\Delta X/\Delta t$) and the middle of the Δt (substituted by t) was shown in Fig. 4.

After ingestion of 0.5 g AM, the time of AM peak concentration in the urine was between 1.0 and 1.5 h. The excretion amount of AM in the urine was 0.2227 g within 6 h. So the excretion level of AM within 6 h was 44.54%.

4. Conclusions

A new analytical procedure based on CE–ECL has been developed for determination of AM and studied its pharmacokinetics in human urine. The proposed method was simple, rapid, economical and sensitive. The method is also suitable to the pharmacokinetics of other lactamic antibiotics in human fluids and animal tissues.

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