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Electrochemiluminescence detection with integrated indium tin oxide electrode on electrophoretic microchip for direct bioanalysis of lincomycin in the urine

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Abstract

In this article, an antibiotic, lincomycin was determined in the urine sample by microchip capillary electrophoresis (CE) with integrated indium tin oxide (ITO) working electrode based on electrochemiluminescence (ECL) detection. This microchip CE–ECL system can be used for the rapid analysis of lincomycin within 40 s. Under the optimized conditions, the linear range was obtained from 5 to 100 μM with correlation coefficient of 0.998. The limit of detection (LOD) of 3.1 μM was obtained for lincomycin in the standard solution. We also applied this method to analyzing lincomycin in the urine matrix. The limit of detection of 9.0 μM was obtained. This method can determine lincomycin in the urine sample without pretreatment, which demonstrated that it is a promising method of detection of lincomycin in clinical and pharmaceutical area.

Keywords: Microchip capillary electrophoresis; Lincomycin

1. Introduction

Lincomycin [methyl 6,8-dideoxy-6-(1-methyl-4-propyl-2-pyrrolidinecarboxamido)-1-thio-D-erythro-α-D-galactooctopyranoside] is a well-established antibiotic drug used in human and veterinary medicine. It is effective primarily against Gram-positive pathogens. Lincomycin has been used in various illnesses, including the mouth and upper respiratory tract infections, as well as skin infections [1,2].

A variety of methods have been used for the analysis of lincomycin, such as microbiological assay method [3,4], chemical assay [5], thin layer chromatography [1], paper chromatography [6] and isotachophoresis [7]. Determination of lincomycin residues in salmon tissues by gas chromatography with nitrogen–phosphorus detection was reported. The limit of detection was 1.7 ppb [8]. The separation of lincomycin from its related substances was reported by a reversed-phase ion-pair liquid chromatographic method with UV detection at 210 nm [9]. Recently, determination of lincomycin in bovine milk, animal muscles and organs using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) was presented [10]. Detection limits of 1.5, 8.8, 4.3, 7.3, 7.8 and 4.7 μg/kg for lincomycin were obtained in the milk, bovine muscle, chicken muscle, bovine liver, chicken liver and bovine kidney, respectively. Capillary electrophoresis (CE) with copper microparticles-modified carbon fiber microdisk array electrodes has been used for the determination of lincomycin. However, the migration time of lincomycin was about 18 min [11].

Electrochemiluminescence (ECL) detection is widely used because of its unique advantages such as simple and

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inexpensive instrumentation, low background noise, high detection sensitivity and a wide dynamic linear range [12]. $Ru(bpy)_3^{2+}$ ECL combination with HPLC, flow injection (FI) [12–14] and conventional CE [15–23] separation has been applied to the determination of oxalates, amino acids, alkylamines, peptides, immunoassays and DNA-probe assays. It can also be used to determine antibiotics [24,25]. Targove and Danielson [24] employed HPLC with $Ru(bpy)_3^{2+}$ ECL method for the determination of clindamycin phosphate and clindamycin, with detection limits of 10 and 100 ppb, respectively. Nieman and co-workers [25] determined erythromycin by microbore liquid chromatography with $Ru(bpy)_3^{2+}$ ECL detection method in the urine and plasma sample. The detection limits for erythromycin in the urine and plasma were 0.05 and 0.1 μM, respectively. Up to now, there has been no report on detection of lincomycin by CE–ECL method.

Very recently, capillary electrophoresis in the microchip format has obtained a great deal of interest because it offers many potential advantages including rapid and efficient separation capability and negligible consumption of solution [26,27]. However, there are few reports on the microchip CE combined with ECL detection [22,23,28]. Manz and his co-workers [22] reported a microfludic system with indirect Ru(phy) ECL detection of amino acids, based on a "U"-shape floating platinum electrode placed across the separation channel. Crooks and his co-workers [28] reported a novel microfluidics-based sensing system that based on EC detection and ECL reporting.

In this paper, lincomycin, a tertiary amine, was determined by the microchip CE–ECL system. In the CE–ECL system, ITO working electrode was fabricated by photolithographic method from an ITO-coated glass slide. The glass slide was served as the chip substrate. The top layer was a poly(dimethylsiloxane) (PDMS) layer, on which there were separation and injection channels. The ITO electrode was aligned at the exit of the electrophoretic separation channel. No decoupler was employed for isolating the high separation electric field in this system because the influence of the high separation voltage on the ECL signal was not significant.

2. Experimental

2.1. Reagents

Tris(2,2′-bipiridine) ruthenium(II) chloride hexahydrate, lincomycin hydrochloride and clindamycin phosphate were purchased from Aldrich (Milwaukee, WI, USA). Sylgard 184 silicone elastomer and curing agent were obtained from Dow Corning (Midland, MI, USA). RZJ-390 (for TN/STN ITO) photoresist was purchased from Suzhou Ruihong Electronic Chemicals Co. Ltd. (Suzhou, China). ITO-coated (150 nm thick and resistance <15 Ω/square) glass was purchased from Hivac Technology Co. Ltd. (Shenzhen, China). Buffer solutions used in the experiment were sodium dihydrogen phosphate and disodium hydrogen phosphate. They were purchased from Beijing Chemical Reagent Factory (Beijing, China). All reagents used were at least analytical reagent grade. All solutions were prepared with distilled water purified by a Milli-Q system (Millipore, Bedford, MA, USA) and stored at 4 °C in a refrigerator. Before use, all solutions were filtered through 0.22 μm membranes.

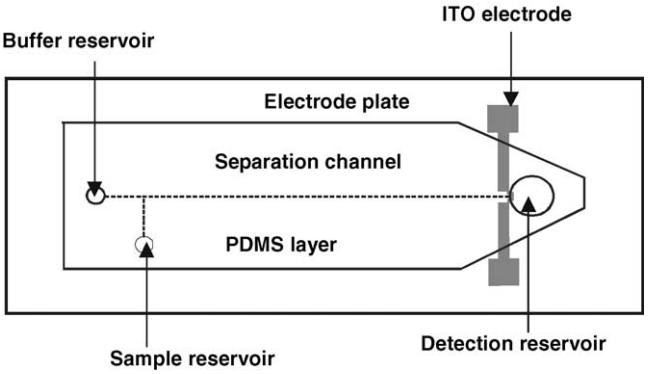

Fig. 1. Schematic diagram of the microchip integrated with ITO working electrode.

2.2. Fabrication

2.2.1. PDMS structures

The method used to fabricate PDMS with designed channels was the same as that reported before [29]. Briefly, a flat soda–lime glass plate, with a thin layer of chromium and positive photoresist, was used as master for PDMS micromolding. A degassed mixture of PDMS monomer and curing agent was pured onto the glass master and cured at 75 °C for 2 h. After it is removed from the mold, the PDMS with negative relief channels was obtained.

2.2.2. Electrode plates

A ITO working electrode was fabricated by wet chemical etching ITO-coated glass slide (7 cm × 2 cm) after a photolithographic procedure of developing photoresist patterns to ITO surface. Details of the electrode fabrication were the same as that of our report earlier [30].

2.2.3. Microchip bonding

After PDMS layer and electrode plate were cleaned with deionized water and ethanol, and dried, they were reversibly bonded together. The working electrode was aligned at the end of the separation channel with the aid of an optical microscope. The schematic diagram of the microchip CE–ECL device was shown in Fig. 1.

2.2.4. Electrophoresis procedure

The electrokinetic sample injection and electrophoretic separation were carried out by a two-channel programmable high-voltage supply (0–2000 V, Remax Electronic Co. Ltd., Xi'an, China). The running buffer used in this experiment

was 20 mM phosphate buffer at pH 5.0. Prior to use, the injection and separation channels were sequentially flushed with 0.1 M NaOH, distilled water and running buffer for 15, 5 and 30 min under vacuum. The sample loading was carried out by applying a 1200 V high potential between the sample and detection reservoirs for 5 s with the detection reservoir grounded. At the same time, 1050 V high voltage was applied at the buffer reservoir. In the separation phase, 1120 V voltage was applied at the sample reservoir and 1200 V voltage at the buffer reservoir with the detection reservoir grounded.

2.2.5. ECL detection

ECL detection was carried out with a Model CHI 800 electrochemical analyzer (CH Instrument, Texas, USA), using a three-electrode cell consisting of a 200 μm ITO working electrode on the glass plate, an Ag/AgCl reference electrode (KCl saturated) and a Pt wire counter electrode. The detection potential was set as 1.2 V. The mixture solution of 10 mM phosphate buffer (pH 7.5) and 5 mM $Ru(bpy)_3^{2+}$ was added to the detection reservoir. ECL signals were collected by a Model MCDR-A Multifunctional Chemistry Analytical Processing System (Xi'an Remex Electronic and Technological Co., Xi'an, China) with the voltage of the photomultiplier tube (PMT) set at 750 V. The principle of the experimental setup was illustrated in the previous paper [30].

3. Results and discussion

Several factors that influence on ECL signals were investigated, including the distance between the electrode and the end of separation channel, pH value of separation buffer and pH value of the buffer in the detection reservoir.

3.1. Effect of the distance between the end of the separation channel and the working electrode

The distance between the end of the separation channel and the working electrode is an important factor that influences the ECL signals according to the mechanism of ECL reaction [31], which was discussed previously [32]. If the end of the separation channel is very close to the electrode, the concentration of $Ru(bpy)_3^{2+}$ around the electrode is diluted. These caused the decrease of ECL intensity (peak height). While the end of the separation channel is far away from the electrode, not all the sample solution from the separation channel reaches the electrode, which will decrease sensitivity [32]. Therefore, in order to achieve high sensitivity, a proper and fixed distance is required. In our work, the working electrode was aligned at the end of the separation channel with the aid of an optical microscope. As it can be seen from Fig. 2, the maximum ECL intensity was obtained when the distance was 40 μm. We selected 40 μm as optimal distance. The deviation in each alignment was less than 2 μm.

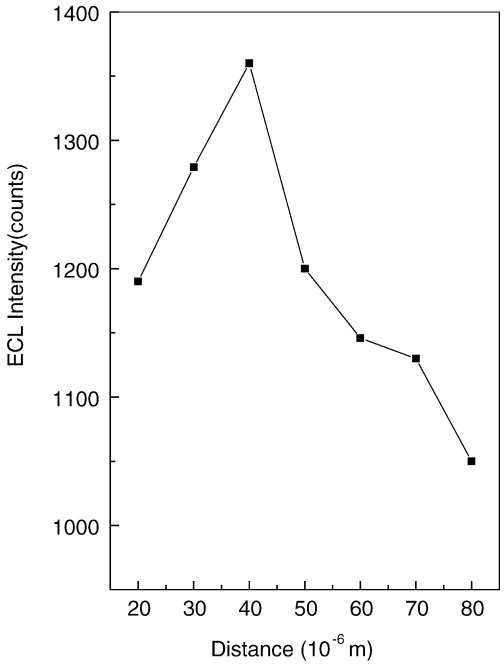

Fig. 2. The effect of the distance between the separation channel and the electrode on the ECL intensity. In injection phase, injection 5 s, 1200 V voltage was applied at the sample reservoir and 1050 V voltage at the buffer reservoir. In the separation phase, 1120 V voltage was applied at the sample reservoir and 1200 V voltage at the buffer reservoir. Sample solution, 100 μM lincomycin; separation solution, 20 mM phosphate buffer (pH 5.0); detection conditions, 5 mM $Ru(bpy)_3^{2+}$ in 10 mM phosphate buffer (pH 7.5); detection potential, 1.2 V (vs. Ag/AgCl).

3.2. Effect of pH value of separation buffer solution

The influence of pH value of separation buffer is of vital importance. When the pH varies, the state of the ionization of analytes in the sample zone and their effective charges change correspondingly [33]. The effect of pH value of separation buffer on the ECL of lincomycin, was investigated from pH 3.0 to 8.0 (Fig. 3). The ECL intensity (peak height) increased with increasing pH from 3.0 to 5.0, and decreased notably from pH 5.0 to 8.0. Therefore, we selected pH 5.0 as the optimal pH value.

3.3. Effect of pH value of buffer solution in ECL reservoir

The pH value is a crucial aspect in the ECL reaction because $Ru(bpy)_3^{2+}$ ECL reaction with alkylamine is a pH-dependent process. ECL signals of alkylamine were maximum in slightly basic conditions [34]. Several groups have studied the effect of pH on the ECL detection for the tertiary amine. The optimum pH for tertiary amine is reported at about pH 8.0–9.0 [35,36]. In this experiment, the ECL intensity (peak height) at various pH values from 6.0 to 10 was investigated. It reached maximum peak at pH 7.5, as illustrated in Fig. 4. This may attribute to the less stability of lincomycin at alkaline condition rather than the stability of $Ru(bpy)_3^{2+}$ [37].

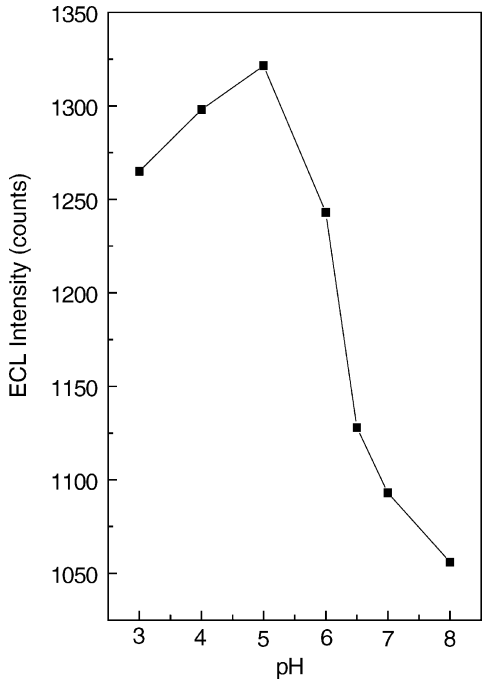

Fig. 3. The effect of the running solution pH on the ECL intensity. Separation solution, 20 mM phosphate buffer. The distance between the separation channel and the electrode was 40 μm. Other conditions were the same as those in Fig. 2.

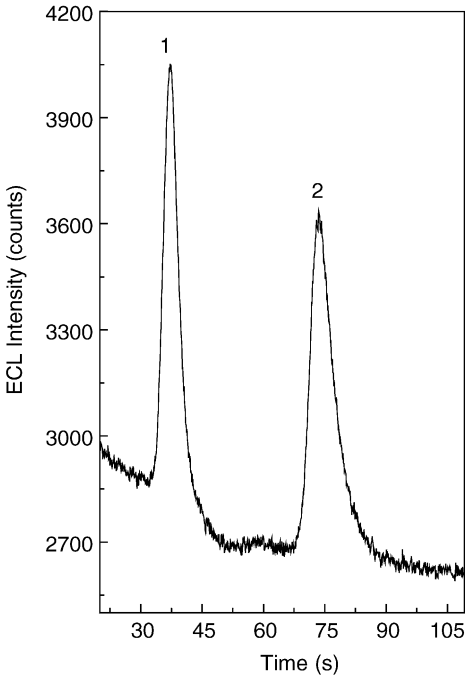

Fig. 5. Electropherograms of a mixture containing 100 μM lincomycin (1) and 100 μM clindamycin phosphate (2). The distance between the separation channel and the electrode was 40 μm. Other conditions were the same as those in Fig. 2.

3.4. Linearity and detection limit

Under optimized conditions: separation solution, 20 mM phosphate buffer (pH 5.0); detection conditions, 5 mM $Ru(bpy)_3^{2+}$ in 10 mM phosphate buffer (pH 7.5); detection potential, 1.2 V (versus Ag/AgCl); the distance between the separation channel and the electrode, 40 μm. The linear range and the detection limit were studied. We investigated the ECL signal with different concentrations of lincomycin (2, 5, 10, 20, 50, 100, 200, 500 μM and 1 mM). The linear range was obtained from 5 to 100 μM with correlation coefficient of 0.998. A limit of detection (LOD) of 3.1 μM (S/N = 3) was obtained. The relative standard deviation of seven replicate injections for 100 μM lincomycin was 5.6, which showed good reproducibility.

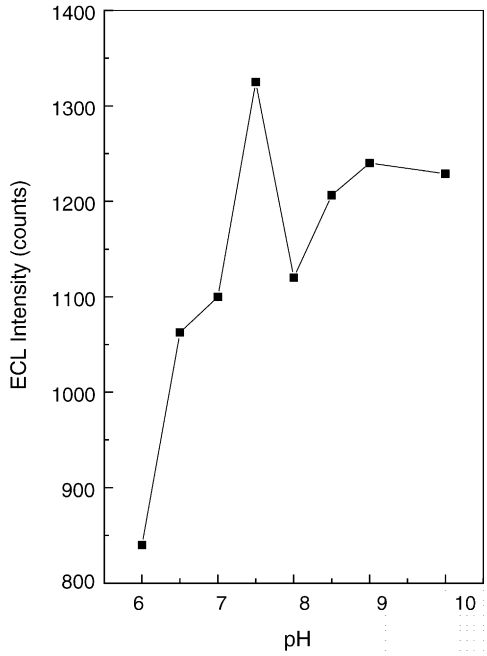

Fig. 4. The effect of the solution pH in the ECL reservoir on the ECL intensity. Detection conditions, 5 mM $Ru(bpy)_3^{2+}$ in 10 mM phosphate buffer. The distance between the separation channel and the electrode was 40 μm. Other conditions were the same as those in Fig. 2.

3.5. The effect of the related antibiotic on lincomycin

Clindamycin phosphate is also an antibiotic of the family of lincosamides. Cross resistance has been demonstrated between lincomycin and clindamycin phosphate. In this CE–ECL system, clindamycin phosphate does not interfere the determination of lincomycin. As we can see from Fig. 5, clindamycin phosphate and lincomycin were well separated under the optimized system within 100 s.

3.6. Analysis of the urine sample

To demonstrate the practical application of the detection of lincomycin by the microchip CE–ECL method, urine samples were analyzed. Prior to analysis, urine samples were filtered through 0.22 μm membranes and then diluted with distilled water by 20-folds to decrease the interference of

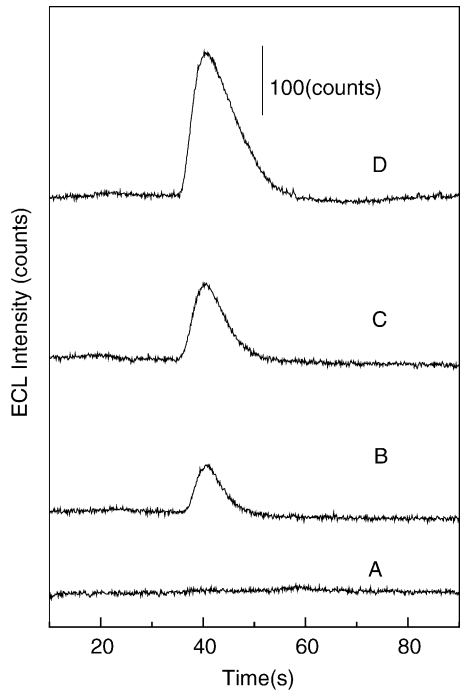

Fig. 6. Electropherograms of: (A) 1:20 diluted blank urine sample; (B) A spiked with 50 μM lincomycin; (C) A spiked with 100 μM lincomycin; and (D) A spiked with 200 μM lincomycin. Other conditions were the same as those in Fig. 5.

Table 1
Recoveries of lincomycin in urine sample with standard addition method

Concentration (μM)	Recovery (%) (n = 5)	R.S.D. (%) (n = 5)
20	90.3	6.5
100	97.1	4.8

the ionic strength of the sample matrix. Fig. 6 showed the electropherogram of the blank urine sample and the typical electropherograms of urine spiked with 50, 100 and 200 μM lincomycin. There was no interference on the ECL detection of lincomycin in the urine. Table 1 showed the recoveries of lincomycin in the urine sample. The recoveries of 20 and 100 μM lincomycin were 90.3 and 97.1, respectively. Under the same optimized conditions as mentioned above, the ECL signal of lincomycin spiked in the urine matrix was lower than that of standard solution. We investigated the analytical performance of this method for determination of lincomycin in the urine matrix, and the results were listed in Table 2. The linearity was from 20 to 200 μM with correlation coefficient of 0.9986. The detection limit was determined to be 9.0 μM (S/N = 3), which was higher than that (3.1 μM) of the standard solution. After taking an administered dose of lincomycin, about 60% metabolized by the liver was excreted through the urine as the unchanged form [38]. In the case of intramuscular injection and intravenous injection, the concentration of injection of lincomycin is higher, the concentration of lincomycin in the urine is higher, too. Thus, we can use this microchip CE–ECL to analyze lincomycin in the urine matrix.

Table 2
Analytical performance of microchip CE–ECL for determination of lincomycin in the urine matrix

Analyte	Lincomycin
Linearity (μM)	20–200
Correlation coefficient	0.9986
Detection limit (μM)	9.0

4. Conclusion

A new microchip CE–ECL system, consisting of an integrated ITO electrode glass plate and a PDMS layer, was characterized by the determination of lincomycin in the urine sample without using the decoupler. Due to its simplicity, selectivity, sensitivity and low sample consumption, microchip CE–ECL has been demonstrated unique advantages in biochemical application. It is a promising method for determination of medicines in clinical analysis.

Acknowledgements

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