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Journal of Chromatography B, 857 (2007) 136–141
JOURNAL OF CHROMATOGRAPHY B
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Determination of erythromycin in rat plasma with capillary electrophoresis–electrochemiluminescence detection of tris(2,2′-bipyridyl) ruthenium(II)

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Received 2 February 2007; accepted 5 July 2007
Available online 24 July 2007

Abstract

A fast and sensitive approach for determination of erythromycin in rat plasma was described. The method used capillary electrophoresis coupled with end-column electrochemiluminescence (ECL) detection of $Ru(bpy)_3^{2+}$. The separation column used had an inner diameter of 75 μm. The running buffer was 15 mmol/L sodium phosphate (pH = 7.5). The solution in the detection cell was 50 mmol/L sodium phosphate (pH = 8.0) and 5 mmol/L $Ru(bpy)_3^{2+}$. ECL intensity varied linearly with erythromycin concentration from 1.0 ng/mL to 10 μg/mL. The detection limit (S/N = 3) was 0.35 ng/mL. The relative standard deviations, of ECL intensity and migration time for eight consecutive injections of 1.0 μg/mL erythromycin ($n = 8$), were 1.3% and 1.8%, respectively. The method was successfully applied to erythromycin determination in rat plasma. The recovery ranged from 92.5 to 97.5%.

Keywords: Capillary electrophoresis; Electrochemiluminescence; Erythromycin; Tris(2,2′-bipyridyl) ruthenium(II); Plasma

1. Introduction

Erythromycin is an important macrolide antibiotic currently used in clinical applications for treatment of bacterial infections. In the last century, erythromycin has been detected both electrochemically [1] and with fluorescence [2,3]. Most methods reported for analysis of erythromycin, in plasma and urine, rely on the use of high performance liquid chromatography (HPLC) [4–7] in addition to capillary electrophoresis (CE) [8]. In recent years, several analytical techniques have been widely studied for erythromycin determination containing HPLC [9,10], voltammentry [11], and detection using ion suppression chromatography [12] and chemiluminescence (CL) [13]. However, low sensitivity and a narrow linear range make it necessary for the development of improved detection techniques for erythromycin and related macrolide antibiotics.

Capillary electrophoresis is an important and powerful analytical separation tool [14]. The advantages of CE include small sample size, high resolution, short analysis time, and low operational cost [15]. Since small sample volumes are used in CE, sensitive detection methods, such as laser-induced fluorescence (LIF), are required. However, the costs associated with LIF limit its application [16]. A viable alternative for CE detection is electrochemiluminescence (ECL) with $Ru(bpy)_3^{2+}$. ECL has many advantages including its simplicity, inexpensive instrumentation, low background noise, high sensitivity, good selectivity, and wide dynamic linear range [15]. In addition, ECL has been used in a variety of applications including detection for biosensors, immunoassays, and flow injection analysis [17].

CE coupled 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 [18–27]. In this work, it was found that weak ECL signals, produced by electrochemical oxidization of $Ru(bpy)_3^{2+}$, were greatly enhanced by erythromycin. Based on this observation, capillary electrophoresis coupled with ECL detection is applied to the determination of erythromycin in rat plasma**.** This method is a highly sensitive, simple, and reliable technique.

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doi:10.1016/j.jchromb.2007.07.010

2. Experimental

2.1. Reagents and chemicals

All reagents used were of analytical grade. Double-distilled water (DDW) was used throughout the study. Erythromycin was obtained from the National Institute for The Control of Pharmaceutical and Biological Products (Beijing, China). Tris(2,2′-bipyridyl) ruthenium(II) chloride hexahydrate was purchased from Alfa Aesar (A Johnson Matthey Company, Ward Hill, MA, USA) and used without further purification. All solutions were prepared with DDW and stored in a refrigerator at 277 K. Working standard solutions were freshly prepared by precise dilution of stock solutions with DDW. The buffer system was Na_2HPO_4/NaH_2PO_4 (Hunan Reagent Company, Hunan, China). Prior to CE analysis, the required sample solutions and phosphate buffer were filtered through 0.45-μm membrane filters (Shanghai Xinya Purification Material Factory, Shanghai, China).

2.2. Apparatus

The CE–ECL system (a high-voltage power supply, an electrical potentiostat, a multifunction chemiluminescence detector and a multichannel data collection analyzer) was produced by Xi'an Remex Electronic Science-Tech Co. Ltd. (Xi'an, China). Output ECL intensity was amplified and recorded in a Pentium 4 PC using the MPI-B software.

The CE–ECL detection cell has been previously described [23]. The end-column ECL cell is composed of a three-electrode system, with a 300 μm-diameter Pt disk as the working electrode, a Pt wire as the counter electrode, and a Ag/AgCl electrode as the reference electrode. The surface of the working electrode was polished with 0.3 μm-alumina powder and cleaned with water in an ultrasonic cleaner before use. A reactivation process, used to eliminate the oxide layer on the Pt electrode, was performed by scanning the applied potential on the Pt disk from −0.5 V to 0.0 V (vs. Ag/AgCl) for 10 cycles. The performance of the working electrode was stable for at least two months following electrochemical treatment. Approximately 300 μL of $Ru(bpy)_3^{2+}$ (5 mmol/L) in phosphate buffer (50 mmol/L, pH 8.0) was placed in the detection cell for ECL measurement. In order to avoid errors in ECL measurements, due to potential changes in $Ru(bpy)_3^{2+}$ concentration over time, the $Ru(bpy)_3^{2+}$–phosphate solution was replaced every 3 h. All electrophoretic experiments were performed using a 40 cm uncoated silica capillary (75 μm i.d., 375 μm o.d.) (Yongnian Optical Fiber Co., Hebei, China). The capillary was filled with 0.1 mol L^{-1} 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.

PHSJ-4A pH meter (Shanghai Precision Science Instrument Co. Ltd., Shanghai, China), SK3200H Ultrasonic Cleaner (Shanghai Kudos Ultrasonics Instrument Co. Ltd., Shanghai, China), TGL-16G-A Centrifuge (Shanghai Anting Science Instrument Factory, Shanghai, China), BF-2000M Nitrogen Blow Instrument (Beijing Bafang Century Technology Co. Ltd., Beijing, China).

2.3. Sample preparation and extraction procedure

Plasma for method development and validation was obtained from rats, weighing 300 g, which were purchased from Guilin Medical College. Polyethylene tubing was inserted and fixed into the femoral vein of rats placed under ether anesthesia. After anesthetic recovery, 1.0 mg/kg of erythromycin was injected intravenously. Blood samples were collected 5 min following administration, immediately heparinized, and centrifuged for 10 min at 3500 rpm in order to separate the plasma. Plasma samples were stored at −20 °C until assay.

Two hundred microlitres of plasma were transferred into a centrifuge tube. After addition of 2 mL of ethyl acetate, the mixture was made alkaline with 5 μL of 1 M NaOH. Mixtures were placed in a mechanical shaker for 5 min and subsequently centrifuged (10 min, 3500 rpm). The upper layer was transferred into another tube and evaporated to dryness under a stream of dry nitrogen at 80 °C. The inner wall of the tube was rinsed with 200 μL of methanol to concentrate the sample. Following solvent evaporation, the remaining dry residue was dissolved in 200 μL of water and measured.

3. Results and discussion

3.1. Optimization of detection conditions

Electrochemiluminescence (ECL) intensity is dependent on the rate of a light-emitting chemical reaction, which is in turn dependent on the potential applied to the electrode. Accordingly, the applied potential must be at least that of the analyte oxidation potential and, consequently, the generated potential of $Ru(bpy)_3^{3+}$. Therefore, the optimal parameter was determined based on ECL intensities at varied potentials. Cyclic voltammograms (CV; Fig. 1(A)) and the corresponding ECL intensities (Fig. 1(B)) were recorded. Under the CV conditions, the rise of erythromycin ECL intensity and buffer solution (Fig. 1(B)) started at about 1.10 V and increased significantly with applied potential. ECL intensity of erythromycin was approximately 10 times higher than the background noise caused by the buffer solution at 1.25 V.

We further investigated the relationship between ECL intensity and applied potential (Fig. 2). This figure illustrates ECL intensity of erythromycin as a function of applied potential from 1.10 to 1.35 V (versus Ag/AgCl). The results correlate with those found in the cyclic voltammetry experiments described above. At lower voltages, a very weak ECL response was obtained. Starting at approximately 1.10 V, the increase in ECL signal intensity becomes readily apparent. The intensity curve reached a plateau and showed the most favorable detection potential at 1.25 V. Therefore, the detection potential was fixed at 1.25 V in the following experiments.

Optimal concentration of $Run(bpy)_3^{2+}$ in the detection cell was an important parameter that has been well studied in previous literature [21]. A low concentration of $Ru(bpy)_3^{2+}$ leads

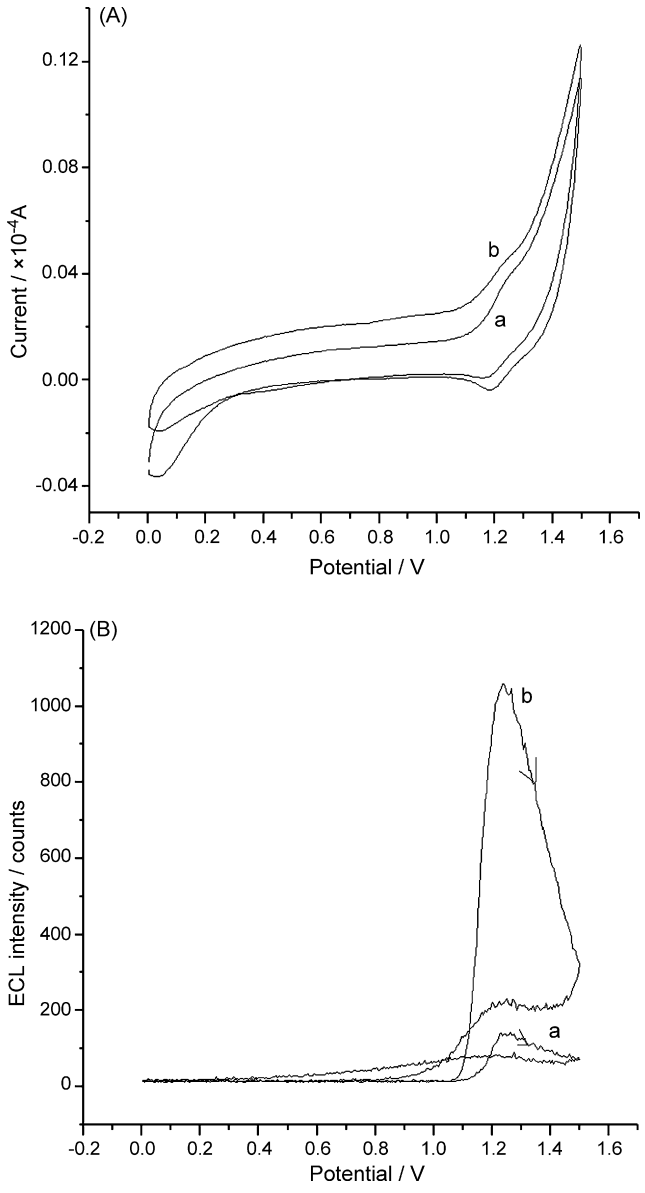

Fig. 1. (A) Cyclic voltammograms and (B) the corresponding ECL intensities. Sample (a) 10 mM Na_2HPO_4/NaH_2PO_4 buffer with pH 7.8 and (b) 1.0 μg/mL erythromycin in water. Conditions: 50 mM buffer with pH 7.8 and 2.5 mM $Ru(bpy)_3^{2+}$ in the ECL cell. Scan rate: 100 mV/s.

to low background noise. On the contrary, detection sensitivity increases with the concentration of $Ru(bpy)_3^{2+}$. Higher concentrations of $Ru(bpy)_3^{2+}$ also produced more noise. Therefore, it is essential to determine a signal-to-noise ratio (S/N) that is optimized with $Ru(bpy)_3^{2+}$ concentrations. In this study, 5 mM $Ru(bpy)_3^{2+}$ was found to produce optimal S/N values and was used in each of the experiments described. 300 μL of $Ru(bpy)_3^{2+}$ solution was determined adequate for over 3 h of operation. Throughout this experiment, the $Ru(bpy)_3^{2+}$ solution was frequently replenished in order to maintain enhanced reproducibility.

The detection buffer pH has a significant effect on ECL response. Previous work has indicated that $Ru(bpy)_3^{2+}$ shows good ECL efficiencies in weak basic solution. This was due to deprotonation of the amine species, forming a reducing free radical intermediate, and the subsequent reaction between the radical intermediate and ruthenium species [28–30,18]. In this study, the effect of buffer pH on erythromycin ECL intensity was determined over a range of pH 6–9 (Fig. 3). Maximum ECL intensity is displayed at pH 8.0. Buffer pH values surrounding 8.0 show decreased ECL intensities. Therefore, an optimal buffer pH of 8.0 was selected for the experimental conditions.

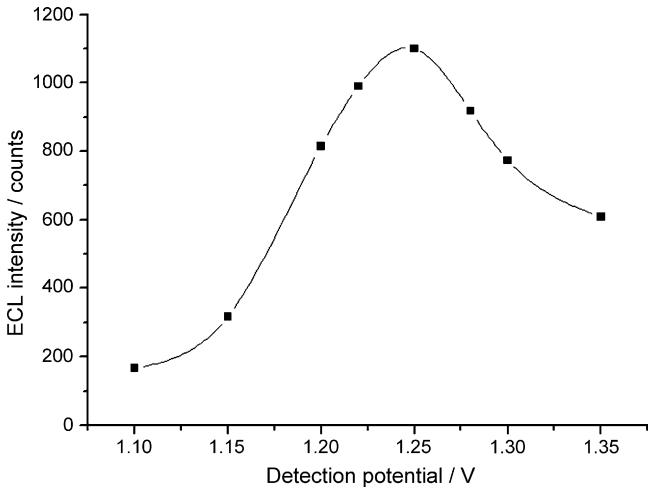

Fig. 2. Effect of detection potential on electrochemiluminescence (ECL) intensity. Conditions: sample, 1.0 μg/mL erythromycin; electrokinetic injection, 10 kV × 6 s; separation buffer, 10 mM (pH 7.8) phosphate buffer; separation voltage, 15 kV; 5 mM $Ru(bpy)_3^{2+}$ and 50 mM (pH 7.8) phosphate buffer in ECL cell.

3.2. Optimization of separation conditions

The pH of the CE buffer plays an important role in CE separation and ECL intensity. This parameter determines electroosmotic flow and analyte charge, collectively influencing migration time. Fig. 4 illustrates the effect of separation buffer pH on detection sensitivity. The ECL intensity of erythromycin

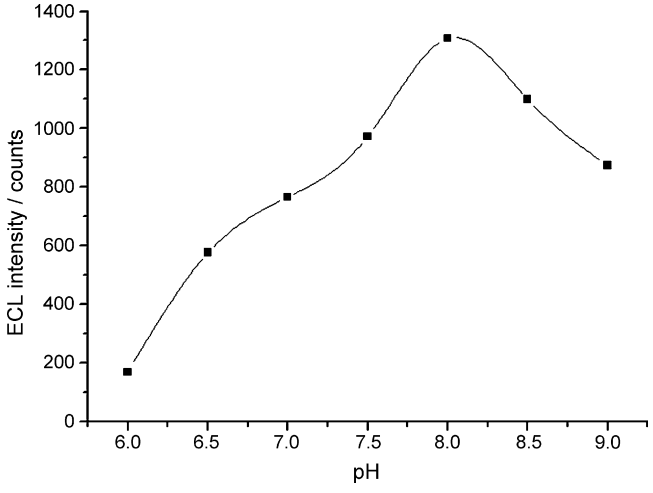

Fig. 3. Effect of the phosphate buffer pH (in ECL cell) on ECL intensity. Conditions: sample, 1.0 μg/mL erythromycin; detection potential, 1.25 V; electrokinetic injection, 10 kV × 6 s; separation buffer, 10 mM (pH 7.8) phosphate buffer; separation voltage, 15 kV; 5 mM $Ru(bpy)_3^{2+}$ and 50 mM phosphate buffer in ECL cell.

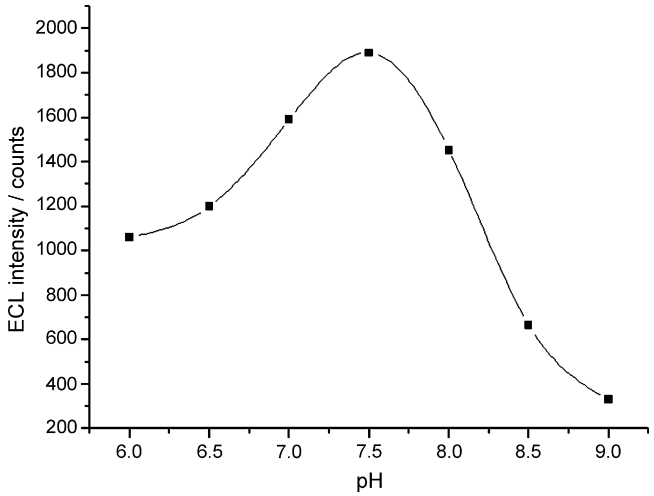

Fig. 4. Effect of separation buffer pH on ECL intensity. Conditions: sample, 1.0 μg/mL erythromycin; detection potential, 1.25 V; electrokinetic injection, 10 kV × 6 s; separation buffer, 10 mM phosphate buffer; separation voltage, 15 kV; 5 mM $Ru(bpy)_3^{2+}$ and 50 mM (pH 8.0) phosphate buffer in ECL cell.

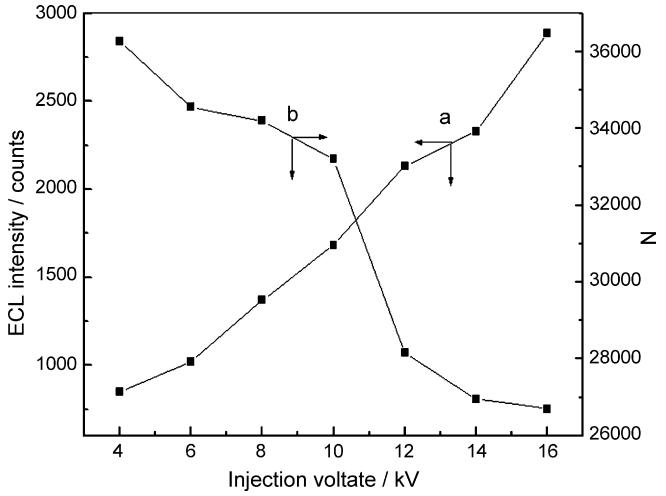

Fig. 6. Dependence of (a) ECL intensity and (b) separation efficiency on the injection voltage. Conditions: sample, 1.0 μg/mL erythromycin; detection potential, 1.25 V; injection time, 6 s; separation buffer, 15 mM (pH 7.5) phosphate buffer; separation voltage, 10 kV; 5 mM $Ru(bpy)_3^{2+}$ and 50 mM (pH 8.0) phosphate buffer in ECL cell.

increased as running buffer pH increased from 6 to 7.5. When the buffer pH exceeded 7.5, ECL intensity decreased and migration time increased. This observation was due to the effect of increasing ionic strength, which results in the increase of Joule heating. At a pH value of 7.5, optimal ECL intensity and migration time were obtained. Therefore, the optimized pH value of running buffer was 7.5.

In this study, ECL intensity also changed as a function of buffer concentration, as illustrated in Fig. 5. An initial increase in ECL intensity was shown as buffer concentration is raised from 5 to 15 mM. Above this concentration, ECL intensity was found to decrease. As a result, 15 mM was determined to exhibit optimal ECL detection and was used as the CE running buffer in subsequent experiments.

The influence of separation voltage on ECL intensity was determined from 8 to 18 kV. High voltages reduce analysis time and sharpen peaks by minimizing zone broadening. Theoretically, resolution and efficiency are directly proportional to the applied voltage [31]. However, the inability of the system to remove excess Joule heat generated at high voltages results in peak broadening and a decrease in efficiency and resolution. Therefore, it is necessary to optimize voltage without compromising resolution. A separation voltage of 10 kV was determined to provide both optimal ECL intensity and good resolution.

3.3. Effect of injection time and voltage

Fig. 6 showed the effect of injection voltage on ECL intensity and theoretical plate number (*N*) at an injection time set at 6 s. Fig. 7 showed the influence of injection time, ranging from 2 to 14 s, on ECL intensity and *N*. Theoretical plate efficiencies of

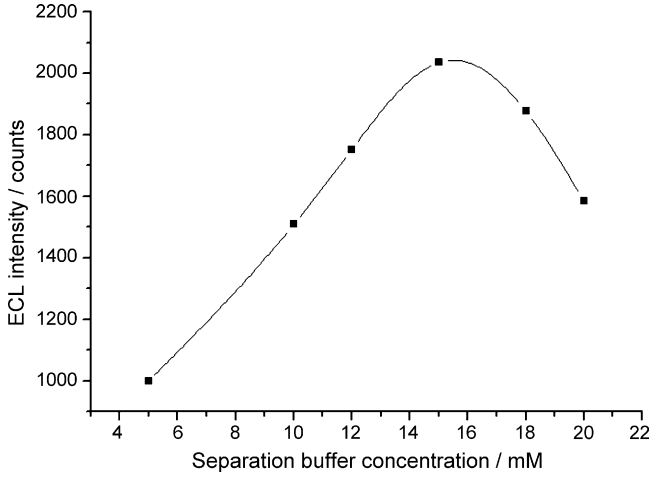

Fig. 5. Effect of separation buffer concentration on ECL intensity. Conditions: sample, 1.0 μg/mL erythromycin; detection potential, 1.25 V; electrokinetic injection, 10 kV × 6 s; separation buffer, pH 7.5 phosphate buffer; separation voltage, 15 kV; 5 mM $Ru(bpy)_3^{2+}$ and 50 mM (pH 8.0) phosphate buffer in ECL cell.

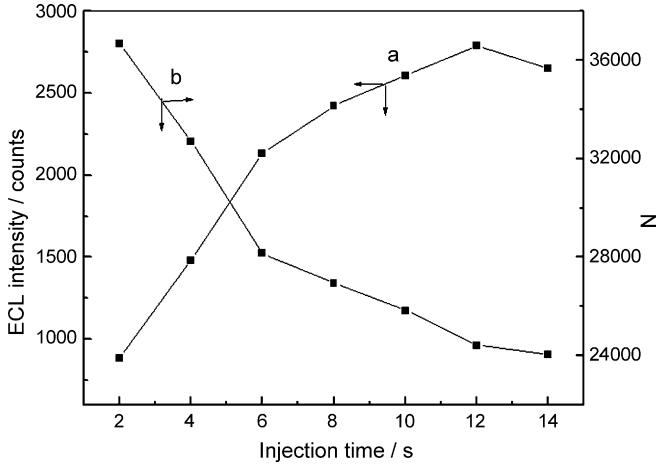

Fig. 7. Dependence of (a) ECL intensity and (b) separation efficiency on the injection time. Conditions: sample, 1.0 μg/mL erythromycin; detection potential, 1.25 V; injection voltage, 12 kV; separation buffer, 15 mM (pH 7.5) phosphate buffer; separation voltage, 10 kV; 5 mM $Ru(bpy)_3^{2+}$ and 50 mM (pH 8.0) phosphate buffer in ECL cell.

Table 1
Analytical results of erythromycin in rat plasma ($n = 5$)

Sample no.	Plasma content (μg/mL)	Added (μg/mL)	Found (μg/mL)	Recovery (%)	R.S.D. (%)
1	1.72	0.400	2.10	95.0	4.4
2	1.80	0.400	2.17	92.5	4.2
3	1.76	0.400	2.15	97.5	3.6

erythromycin were calculated using the equation $N = 16(t_R/W)^2$, where t_R is the retention time and W is the bottom width of the peak. As illustrated in Figs. 6 and 7, long injection times and high voltages resulted in strong ECL signals and low separation efficiencies. This is due to the introduction of additional analyte into the detection cell. However, when shorter injection times and lower injection voltages were used, high column efficiencies were obtained at the expense of optimal ECL intensities. An electrokinetic injection duration of 8 s at 12 kV was determined to provide an optimal compromise amongst the studied parameters [32].

3.4. Linearity, detection limit and reproducibility

The optimized experimental conditions consist of a 40 cm separation capillary (75 μm i.d.), 1.25 V applied potential on the Pt disc of the ECL detection cell, 5 mM $Ru(bpy)_3^{2+}$ and 50 mM phosphate buffer (pH 8.0) in the detection cell, 10 kV separation voltage, 8 s injection time, 12 kV injection voltage, and 15 mM running buffer (pH 7.5). The calibration curve for erythromycin is linear over the concentration range from 0.001 to 10 μg/mL with a regression curve of $y = 14678x + 225.9$ (y for peak area, x unit for μg/mL, $r^2 = 0.9988$). The detection limit was 0.35 ng/mL with a signal-to-noise ratio of 3. The relative standard deviations (R.S.D.) of the ECL intensity (peak areas) and the migration time for eight consecutive injections of 1.0 μg/mL erythromycin were 1.3 and 1.8%, respectively.

3.5. Application

The proposed CE–ECL method was used for the determination of erythromycin in rat plasma samples. The complexity of the plasma matrix can influence the results obtained throughout sample analysis. Since an electrokinetic injection mode was utilized in this study, the ionic strength of the sample matrix could influence the injection of samples. In addition, some organic compounds presented in the plasma can influence the ECL reaction. Therefore, an extraction procedure was performed to remove ions and some organic compounds in plasma in order to obtain a clear electrophoretic sample profile, high detection sensitivity, and good reproducibility [33]. Electrophoregrams obtained from the extraction procedure are illustrated in Fig. 8(a) and (b). Erythromycin was determined to be an average of 1.76 μg/mL in plasma. Table 1 shows the recoveries of erythromycin spiked in plasma samples. The recoveries of erythromycin in plasma samples ranged from 92.5 to 97.5%. The R.S.D. of ECL peak intensity was less than 5%.

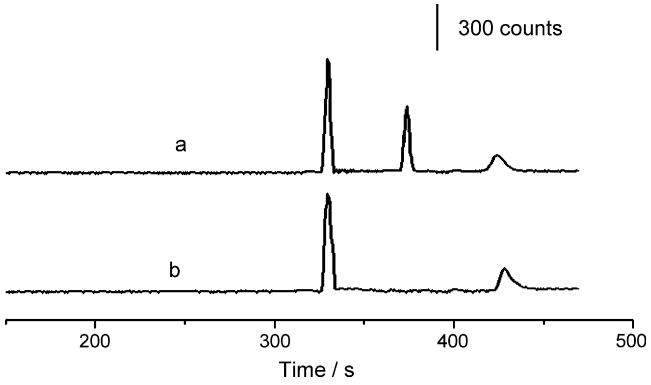

Fig. 8. Electropherograms of (a) spiked plasma sample and (b) blank plasma. Conditions: detection potential, 1.25 V; electrokinetic injection, 8 s at 12 kV; 15 mM separation buffer (pH 7.5); 50 mM (pH 8.0) buffer in the ECL cell; $Ru(bpy)_3^{2+}$, 5 mM; separation voltage, 10 kV.

4. Conclusions

A new analytical procedure based on CE–ECL has been developed for determination of erythromycin in rat plasma. The proposed method is simple, quick, economical, and sensitive. ECL intensity varied linearly with the erythromycin concentration in the range of 1.0 ng/mL to 10 μg/mL. The detection limit (S/N = 3) was 0.35 ng/mL. The relative standard deviations of ECL intensity and migration time, for eight continuous injections of 1.0 μg/mL erythromycin ($n = 8$), were 1.3 and 1.8%, respectively. This method was successfully applied to the determination of erythromycin in rat plasma.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 20565001) and the Guangxi Science Foundation of China (Grant No. 0639029).

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