Electrochimica Acta 54 (2009) 2379–2384

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Tris(2,2′-bipyridyl) ruthenium(II)–bisoprolol-based electrochemiluminescence coupled with capillary zone electrophoresis

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ARTICLE INFO

Article history:
Received 11 September 2008
Received in revised form 20 October 2008
Accepted 25 October 2008
Available online 12 November 2008

Keywords:
Capillary zone electrophoresis with end-column electrochemiluminescence (CZE–ECL)
Tris(2,2-bipyridyl) ruthenium(II)
Beta-blockers
Bisoprolol
Metoprolol
Drug analysis

ABSTRACT

Capillary zone electrophoresis (CZE) coupled with tris(2,2′-bipyridyl) ruthenium(II)-based end-column electrogenerated chemiluminescence (ECL) has been utilized to detect bisoprolol in drugs and tablets after its separation from metoprolol. Tetrahydrofuran was used as an additive in the running buffer to obtain the absolute ECL peak of bisoprolol. Bisoprolol reacts as a *co-reactant* in tris(2,2′-bipyridyl) ruthenium(II) ECL system. Under the optimized experimental conditions, bisoprolol was separated successfully and efficiently from metoprolol and other co-existed materials in tablets and urine samples. The ECL intensity of tris(2,2′-bipyridyl) ruthenium(II)-bisoprolol-based system is linear with the concentration of bisoprolol from 1.5 μM to 0.3 mM with a detection limit of 0.3 μM. Relative standard derivations of the ECL intensity are 2.58% for the detection of 15 μM bisoprolol. This method is a simple, rapid, selective, and sensitive. It was applied successfully for the monitoring of bisoprolol in market available tablets and human urine samples.

1. Introduction

Capillary zone electrophoresis coupled with electrogenerated chemiluminescence (CZE–ECL) has attracted much attention recently as a powerful and popular analytical/separation technique. Capillary zone electrophoresis separates molecules by a capillary with small inner-radius under the command of an electric field, while electrogenerated chemiluminescence is utilized as a detection tool [1–12]. CZE–ECL shows characteristics of high resolving power, efficiency, flexibility, accuracy but small amount of sample solutions required for injection. It has been applied widely to determine various analytes like pharmaceutical compounds [2,3], DNA, and proteins [4]. Considerable attention has been specially focused on the application of CZE–ECL for the separation and detection of analytes containing tertiary amine groups and their derivatives [11–16].

Beta-blockers (like bisoprolol and metoprolol) belong to amine derivatives and have quite important biological functions. However, relatively few works paid attention to the application of CZE–ECL for the detection of beta-blockers. Bisoprolol is a highly selective β_1 adrenoceptor antagonist for the treatment of coronary heart disease and hypertension [17,18]. The S(-) enantiomer of bisoprolol is responsible for most of the beta-blocking activity and effective in reducing blood pressure. It has also shown beneficial cardiac effects in patients with hypertension [19,20]. The full chemical name of bisoprolol is ±1-[4-[[2-(1-methylethoxyl) ethoxyl] methyl] phenoxy]-3-[(1-methylethyl) amino]-2-propanol, as shown in Fig. 1. Metoprolol (shown in Fig. 1) is prescribed as a substitute for β_1 adrenoceptor blocking drug. Several methods for monitoring bisoprolol and metoprolol in pharmaceutical preparations, plasma, serum and urine samples have been developed [21–29]. Most of them rely on chromatographic techniques [21–26] or capillary electrophoresis [27,28]. Voltammetric detection by single-walled carbon nanotube modified glassy carbon electrode [29] and electrospray ionization-mass spectrometric detection [24] have also been reported. However, narrow linear ranges, high detection limits, time-consuming extraction procedures, expensive instrumentations, and high running costs limit the practical applications of these methods.

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doi:10.1016/j.electacta.2008.10.066

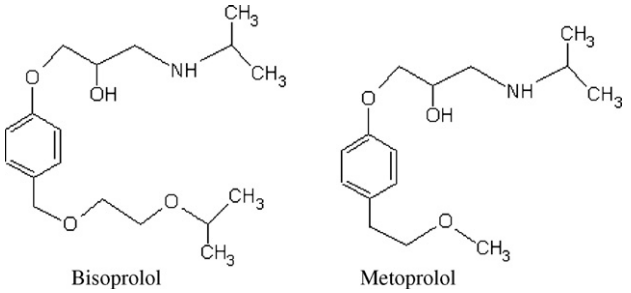

Fig. 1. Molecular structures of bisoprolol and metoprolol.

In this work, we report the application of CZE–ECL for the detection of bisoprolol in tablets and urine samples after its separation from metoprolol and other co-existed materials. Tris(2,2′-bipyridyl) ruthenium(II)-based ECL system was utilized as it shows advantages of high sensitivity, good selectivity, a wide dynamic linear range, simplicity of operation, and low cost for instrumentation. The experimental conditions for the separation of bisoprolol from metoprolol and for their ECL detection were optimized. Tetrahydrofuran (THF) was used as an additive in the running buffer since it can remove the interference and finally result in the absolute ECL peak of bisoprolol [30,31]. The application of the proposed method for the detection of bisoprolol and metoprolol in commercial drugs and in human urine was also conducted.

2. Experimental

2.1. Chemicals and apparatus

Tris(2,2′-bipyridyl) ruthenium(II) chloride hexahydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Bisoprolol and metoprolol were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Nanchang, China). Bisoprolol fumarate tablets were from Merck KGA Darmstadt in Germany. Other chemicals are of analytical-reagent grade. The pH values of buffer solutions were adjusted with 1.0 M sodium hydroxide. The stock solutions of 300 μM bisoprolol and 200 μM metoprolol in pH 8.0 phosphate buffer solutions were stored in the refrigerator at 4 °C. Sample solutions were prepared by diluting stock solutions with phosphate buffers just before measurements. The running buffer was mixed with THF in a volume ratio of 98:2. Water was purified in a Milli-Q System (Millipore, Bedford, MA, USA). Before CZE analysis, sample solutions and phosphate buffers were filtered through 0.22-μm membrane filters (Shanghai Xinya Purification Material Factory, Shanghai, China).

The CZE–ECL detection system was bought from Xi'an Remax Electronics Co. Ltd., Xi'an, China. A fused-silica capillary (25 μm i.d., 375 μm o.d.) was purchased from Yongnian Optical Fabric Factory (Hebei, China). A Model CHI600 voltammetric analyzer (CH Instruments, Austin, TX, USA) was used for electrochemical experiments. A three-electrode system was used with a 300-μm platinum disk as the working electrode, an Ag/AgCl as the reference electrode (in saturated KCl solution), and a platinum wire as an auxiliary electrode. The ECL cell was constructed as described previously [32,33].

2.2. Procedure

Before CZE experiments, the capillary has to be flushed continuously with 0.1 M NaOH overnight, then with double distilled water, and lastly with the running buffer until the baseline of ECL is flat at the first use.

ECL detection was carried out in an end-column mode of reaction. In order to make CZE effluent contact with the electrode surface directly, the distance between the working electrode and the outlet of the capillary was controlled strictly in the range of 70 ± 5 μm [32–36] with the aid of an optical microscope. The inlet end of the capillary was held at a positive potential, and the outlet end was maintained at ground by a stainless tube which was fixed on the capillary with epoxy glue. The analytes were injected by electrokinetic mode at 10 kV for 10 s. A photomultiplier tube (PMT) positioned under the detection cell was set at −800 V to collect the ECL signals. The output of ECL intensity was amplified and recorded with MPI-B software by a computer. Required amount of 5 mM $Ru(bpy)_3^{2+}$ in the 50 mM phosphate buffer was filled in the cell during the experiments and refreshed every 2 h.

Prior to ECL measurements, the Pt disc working electrode was polished with 0.3 and 0.05 μm Al_2O_3 slurry on silk cloth in sequence, and cleaned with distilled water in an ultrasonic cleaner. Elimination of the oxide layer on the Pt electrode was always performed *via* scanning the potential from −0.5 to 0.0 V for 10 cycles. 250 μL of $Ru(bpy)_3^{2+}$ solution (pH 8.0) was added into the reservoir before analysis, and replaced every 2 h to eliminate depletion effect or potential interference from reaction during the analysis. Extraction was done before electrophoresis to eliminate the influence of ionic strength in sample and obtain clear electrophoretic profile. Injections were performed by electromigration at 10 kV for 10 s. During the experiments, a 10 kV separation voltage was applied through the capillary, the potential of the ECL detection was 1.17 V and the potential of PMT was set at −800 V.

For the monitoring of bisoprolol and metoprolol, the following experimental conditions were adopted: ECL system, 5.0 mM $Ru(bpy)_3^{2+}$ in 50 mM pH 8.0 phosphate buffer; applied potential on Pt electrode, 1.17 V; separation voltage, 10 kV; running buffer, 10 mM pH 8.1 phosphate buffer; injection voltage, 10 kV; injection time, 10 s; THF concentration, 2% (v/v).

2.3. Sample treatment

The urine samples were collected from volunteers in our laboratory who were orally administrated to tablets containing 5 mg bisoprolol fumarate with empty stomach according to the usage and dosage of the enclosed leaflet. Their urine samples were periodically collected every 2 h and summed to four times. We followed the extraction procedure by Wu et al. [37,38] after a minor modification. Briefly, urine sample (1.0 mL) was injected into a clean 2.0-mL Eppendorf tube, and 1.0 mL extraction solvent (heptane/ethyl acetate = 90:10, v/v) was added into this Eppendorf tube and the tube was sealed. Then, the urine sample was mixed by a medium motion on a shaker for 2 min and then centrifuged at 2000 rpm for 10 min. The separated organic layer at the top was transferred into a new Eppendorf tube. This procedure was repeated three times. The organic layers at the top were then evaporated to dry under a gentle steam of nitrogen at 35 °C in a water bath. The dry residue was reconstituted with 500 μL water and centrifuged for 60 s. Finally the sample solution was injected into the electrophoresis system by electrokinetic injection for 10 s at 10 kV(about 5.0 nL) for the concentration detection.

3. Results and discussion

3.1. ECL of $Ru(bpy)_3^{2+}$ in the presence of bisoprolol and metoprolol

Fig. 2 shows voltammetric response of 5.0 mM $Ru(bpy)_3^{2+}$ in 50 mM pH 8.0 phosphate buffer at the Pt electrode before (a) and

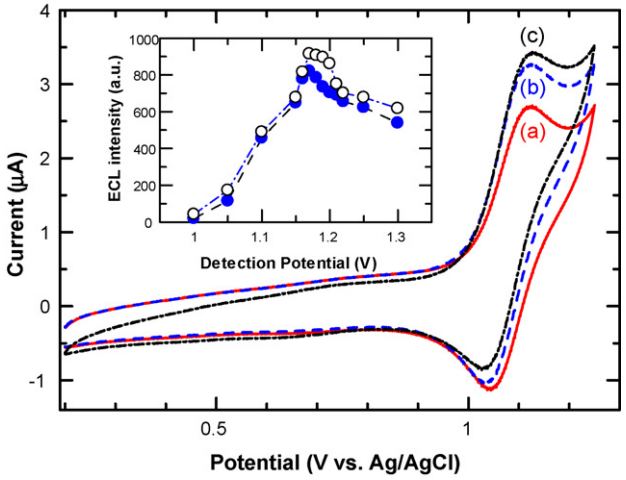

Fig. 2. Cyclic voltammograms of 5.0 mM $Ru(bpy)_3^{2+}$ in 50 mM pH 8.0 phosphate buffer before (a) and after adding 10 μM bisoprolol (b) or 10 μM metoprolol (c) at a scan rate of 0.1 V s^{-1}. The insert is the intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system (full circles) and $Ru(bpy)_3^{2+}$–metoprolol ECL system (open circles) as a function of applied potentials on the Pt working electrode.

after adding 15 μM bisoprolol (b) or after adding 10 μM metoprolol (c). Adding bisoprolol (b) or metoprolol (c) leads to an increase in anodic peak current but a decrease in cathodic peak current. The magnitude of anodic peak currents is much larger than the cathodic ones, indicating the participation of bisoprolol and metoprolol as a *co-reactant* in $Ru(bpy)_3^{2+}$-based ECL system. The insert of Fig. 2 shows the measured intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system (full circles) and $Ru(bpy)_3^{2+}$–metoprolol ECL system (open circles) as a function of applied potentials on Pt electrode. In both systems, the intensities are enhanced with a positive increase of applied potential, and reached maximum values at the peak potentials of bisoprolol and metoprolol from cyclic voltammograms. Higher potentials than their peak potentials result in weakening the ECL intensity. Similar behavior has also been noticed at glassy carbon electrodes. The source of this behavior is not clear for us and under investigation currently in our lab. The potential of 1.17 V was then applied in the following experiments for the detection of bisoprolol and metoprolol.

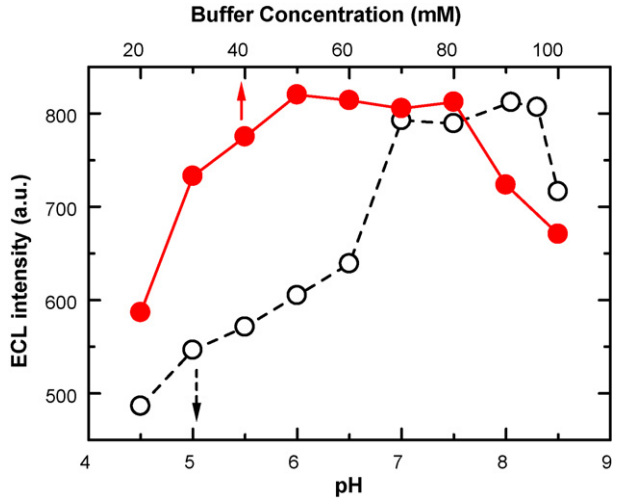

Fig. 3. The intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system as a function of pH values (open circles) and the concentration of phosphate buffer (full circles). The concentration of $Ru(bpy)_3^{2+}$ was 5.0 mM and the applied potential was 1.17 V.

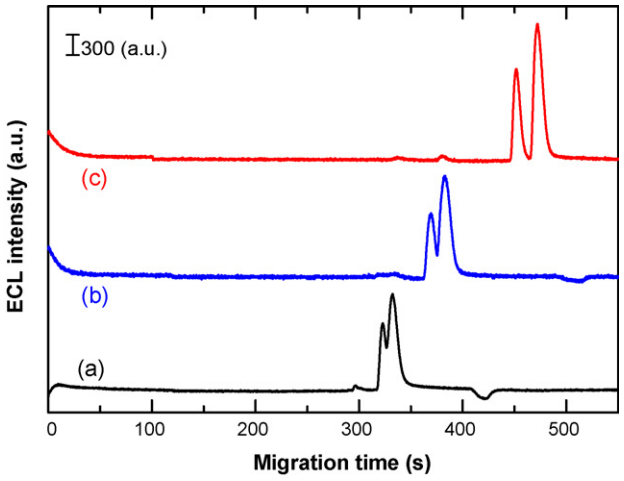

Fig. 4. Electropherograms of the mixture of 15 μM bisoprolol (the peak at right side) with 10 μM metoprolol (the peak at left side) at different separation voltage of 12 kV (a), 11 kV (b), and 10 kV (c).

The pH value and the concentration of phosphate buffer are well-known to be important during ECL reaction process [39–44]. Fig. 3 shows the intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system as a function of pH values (full circles) and the concentration (open circles) of phosphate buffer. The ECL intensity increases with pH value from 4.5 to 8.0 and then decreases when the pH value of buffer was larger than 8.0. The decrease of the ECL intensity probably results from the chemiluminescent reaction of $Ru(bpy)_3^{3+}$ with the high energy intermediate, $HO_2^{\bullet}$ [41–43], electrogenerated in the alkaline solution. Maximum ECL intensity is obtained when the pH value is 8.0 with a concentration range from 50 to 80 mM. Similar effects of pH values and the concentration of phosphate buffer on the intensity of $Ru(bpy)_3^{2+}$–metoprolol ECL system have been noticed. In the following sections, 50 mM pH 8.0 phosphate buffer was utilized for the detection of bisoprolol and metoprolol.

3.2. Separation of bisoprolol and metoprolol by CZE

Fig. 4 shows the electropherograms of the mixture of bisoprolol and metoprolol at different separation voltages. When the separation voltage is set as 10 kV (c), two clear and separated peaks are noticed, indicting the efficient separation of bisoprolol from metoprolol. In curve (c), the peak with a migration time of 452 s at the left side results from metoprolol and the peak with a migration time of 476 s (7.9 min) at the right side results from bisoprolol. The migration time (7.9 min) of bisoprolol is shorter than the reported CZE–UV (24 min) [28] as well as HPLC (8.3 min) [24], indicating a rapid separation. The difference of their migration time is about 20 s. When higher separation voltage than 10 kV is applied (a and b), the migration times of bisoprolol (the peak at right side) and metoprolol (the peak at left side) do become shorter but the noise of the baseline becomes larger. The enlargement of the baseline is probably caused by the increase of Joule heating in the capillary when high voltage is applied. Moreover, the difference of their migration time became smaller, indicating that high separation voltage will fail in the separation of bisoprolol from metoprolol. The resolution of the separation of bisoprolol from metoprolol is evaluated by the following equation [45,46]:

$$R_s = \frac{2(t_2 - t_1)}{W_{b,1} + W_{b,2}} \quad (1)$$

where t_1 and t_2 are the migration times of bisoprolol and metoprolol, respectively, $W_{b,1}$ and $W_{b,2}$ are their peak widths at half-height

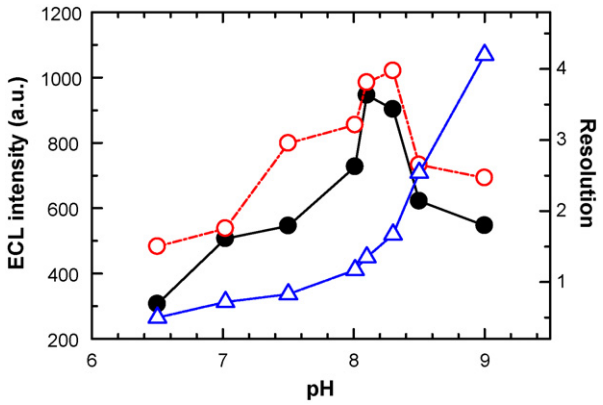

Fig. 5. The intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system (open circles) and $Ru(bpy)_3^{2+}$–metoprolol ECL system (full circles) as well as the resolution of the separation of bisoprolol from metoprolol as a function of pH values of running buffers. For CZE, 10 kV separation voltage was applied. For ECL measurements, 5.0 mM $Ru(bpy)_3^{2+}$ in 50 mM pH 8.0 phosphate buffer was used and the applied potential on Pt electrode was 1.17 V.

of analytes. The calculated resolution as well as the ECL intensity for bisoprolol and metoprolol decreased with an increase in injection voltage. The separation voltage of 10 kV was then selected after taking migration time, and the resolution of bisoprolol from metoprolol for the separation into account.

The effects of the pH value and the concentration of running buffer, injection time and injection voltage on the separation as well as on the ECL intensity were examined. Fig. 5 shows the intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system (full circles) and $Ru(bpy)_3^{2+}$–metoprolol ECL system (open circles) as a function of pH value of the running buffer. The ECL intensities for both ECL systems are enlarged with an increase of pH values, reached maximum vales when pH value is 8.1, and then start to decrease when pH values are higher than 8.1. However, the resolution keeps increasing with increased pH values. It is known that the pH value of the running buffer plays an important role in CE for its influence on zeta potential, the electroosmotic flow (EOF) as well as the charge of all analytes. In basic media, metoprolol and bisoprolol are possibly facile to be ionized and the charged metoprolol and bisoprolol molecules will enhance their electrophoretic mobilities, leading to

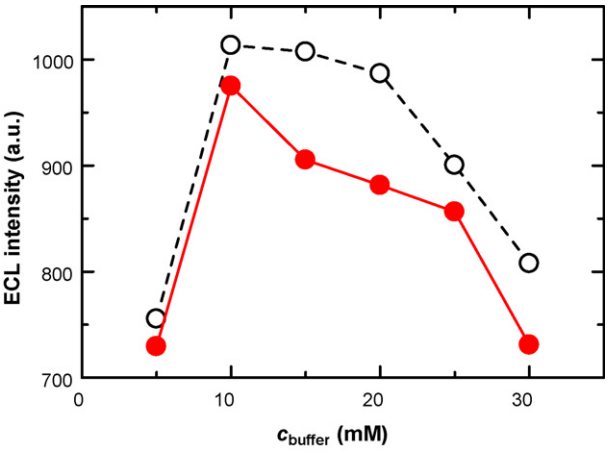

Fig. 6. Effects of the concentration of running buffer on the intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system (open circles) and $Ru(bpy)_3^{2+}$–metoprolol ECL system (full circles). For CZE, 10 kV separation voltage was applied and pH 8.1 phosphate buffer was used as running buffer. For ECL measurements, 5.0 mM $Ru(bpy)_3^{2+}$ in 50 mM pH 8.0 phosphate buffer was used and the applied potential on Pt electrode was 1.17 V.

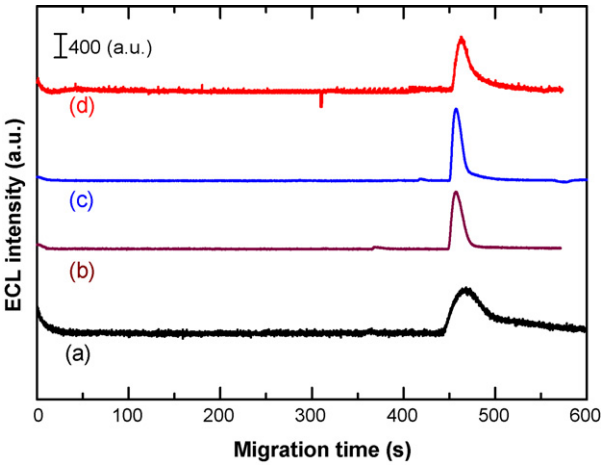

Fig. 7. Effects of 0% (a) 1% (b), 2% (c), and 5% (d) THF (v/v) on the electropherogram of bisoprolol. Experimental conditions were: bisoprolol, 15 μM; detection voltage, 1.15 V; electrokinetic injection, 10 s at 10 kV; running buffer, 10 mM pH 8.1 phosphate buffer; separation voltage, 10 kV.

an increase of resolution value. Therefore, pH 8.1 phosphate buffer was adopted.

The concentration of buffer affected the intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system (full circles) and $Ru(bpy)_3^{2+}$–metoprolol ECL system (open circles). The results are shown in Fig. 6. The intensities of both ECL systems increase when the buffer salt concentration is changed from 5 to 10 mM, reach maximum value when the salt concentration is 10 mM, and then decrease with a further increase of salt concentration. Meanwhile, the half-peak widths of bisoprolol and metoprolol in their electropherograms decrease when higher slat concentrations are used. Thus 10 mM pH 8.1 phosphate buffer was selected as running buffer for the monitoring of bisoprolol and metoprolol.

Tetrahydrofuran (THF) is known as an excellent polar regulator for solvent and widely added into chromatographic system as an additive to improve the separation resolution (R_s) and migration time of analytes (t_m) [30,31]. The effect of the addition of THF into the running buffer on the separation of bisoprolol from metoprolol and on their migration time was investigated and the results are shown in Fig. 7. The addition of THF into running buffer affects the shape of ECL peaks. As shown in curve (a) in Fig. 8, the peak shape in the electropherogram is quite broad and the ECL intensity is relatively small when no THF is present in running buffer. An increase of THF concentration to 2% (v_{THF}/v_{buffer}) (curve c) results in sharp peak and strong ECL intensity. Further increase in the concentra-

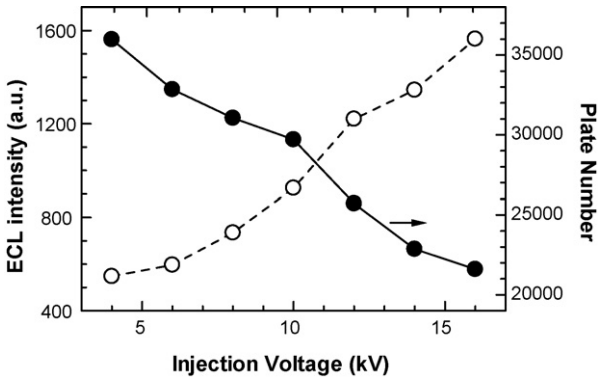

Fig. 8. Effects of injection voltage on the intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system (open circles) as well as on calculated plate numbers (full circles).

Table 1
Determination of bisoprolol in tablet.

Number	Labeled (mg/tablet)	Found[a] (mg/tablet)	Recovery (mg/tablet)	R.S.D. (mg/tablet)	Measured by UV[a] (mg/tablet)
H20060100	5	5.1	102	2.85	5.2
H20060101	2.5	2.49	99.6	2.78	2.51

[a] Average value of six measurements.

Table 2
Determination of bisoprolol in human urine samples.

No. of urine samples	Found[a] (μM)	Added (μM)	Total[a] (μM)	Recovery (%)	R.S.D.
1 (after 2 h)	1.4	3	4.32	98.2	2.78
2 (after 4 h)	2.2	3	5.30	102	2.73
3 (after 6 h)	0.7	3	3.65	98.6	2.84
4 (after 8 h)	0.4	3	3.31	97.4	2.89

[a] Average value of six measurements.

tion (curve d), on the contrary, broadens the peak and weakens the ECL intensity. The enhancement of noise and peak tailing are also noticed when larger concentration of THF (5%) is used (curve d). Therefore, 2% THF (v/v) was added into running buffer in the following experiments.

Fig. 8 summarizes the effect of injection voltage on the intensity of $Ru(bpy)_3^{2+}$–bisoprolol ECL system (open circles) as well as on calculated plate numbers (full circles). Higher injection voltage results in stronger ECL intensity. Similarly, longer injection time results in enhancement of ECL intensity (not shown). On the other hand, the number of theoretical plates, *N*, which can be calculated according to the following equation [47]:

$$N = 5.54\left(\frac{t_m}{W_{1/2}}\right)^2 \quad (2)$$

where t_m is the migration time and $W_{1/2}$ is the width at half height of the electrophoretic peak, showed that *N* decreases with an increase of injection voltage (full circles) and injection time (not shown) for both of $Ru(bpy)_3^{2+}$–bisoprolol ECL system and $Ru(bpy)_3^{2+}$–metoprolol ECL system. It has been known that the ECL intensity mainly depends on the concentration of analyte in the diffusion layer. Driven by higher injection voltage, larger amount of analytes are involved ECL reaction, resulting in higher ECL signal. However, the dispersion of analytes also broadens peaks and decreases *N*. After considering the detection sensitivity and the CE efficiency, we used an injection voltage of 10 kV with an injection time of 10 s for the further experiments

3.3. CZE–ECL for the detection of bisoprolol and metoprolol

Under these optimized conditions, the mixtures of bisoprolol and metoprolol with different concentrations were injected and the electropherograms were recorded. Fig. 9 shows typical examples of electropherograms of 15 μM bisoprolol (dashed line) and 10 μM metoprolol (dotted line) as well as the mixture (solid line) of 10 μM metoprolol and 15 μM bisoprolol. Bisoprolol shows a migration time of 476 s (dashed line) and metoprolol shows a migration time of 452 s (dotted line). Therefore, the peak (a) at 452 s in the electropherogram of the mixture results from metoprolol and the peak (b) at 476 s results from bisoprolol. These results clearly show the efficient separation with quite stable and measurable ECL intensities. The ECL intensity, *I*, is linear with the concentration, *c*, of analytes. The linear range is 1.5 μM to 0.3 mM for bisoprolol with a detection limit of 0.3 μM. The regression equation is $I = 1124.71 + 8.22 \times 10^6 c$ ($r = 0.9936$). For metoprolol, the linear range is 2.0 μM to 0.6 mM, the detection limit is 0.4 μM, and the regression equation is $I = 703.54 + 13.21 \times 10^6 c$ ($r = 0.9934$). The proposed method shows lower detection limits and sensitive detection than those reported in literatures [27,29].

The proposed method was applied to monitor the concentration of bisoprolol in commercial drugs and in urine samples. Under the optimized conditions, the separation of bisoprolol from other materials existing in tablets and urine samples was firstly conducted. Fig. 10 shows electropherograms of urine sample without roxithromycin (curve a), 3 μM bisoprolol standard solution (curve b), urine samples after administration for 4 h (curve c), and the mixture of urine samples after administration for 4 h with standard bisoprolol solution. The peaks at around 470 s are from bisoprolol and other peaks are from impurities of samples. Neither shift on migration time nor the occurrence of additional peaks is noticed, indicating no interference from the co-existed species (matrix materials) in urine sample. Tables 1 and 2 list the detection results of bisoprolol in tablets and urine samples by the use of proposed method, respectively. As control experiments, the concentrations were also measured by the traditional UV method (λ = 272 nm). The concentrations measured by our method agree well with those from UV method. The interferences in urine samples do not affect the monitoring of bisoprolol after separation by CZE. The recovery is in the range of 97–102% and the R.S.D. for these measurements is in

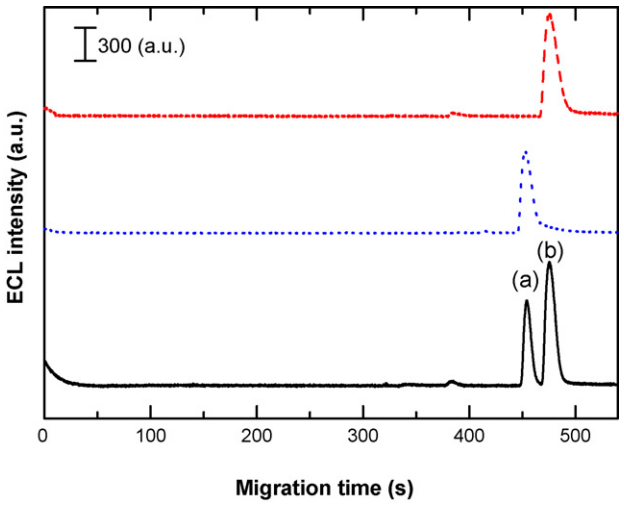

Fig. 9. Electropherogram of 15 μM bisoprolol (dashed line), 10 μM metoprolol (dotted line), and the mixture (solid line) of 10 μM metoprolol (peak a) and 15 μM bisoprolol (peak b). The following experimental conditions were adopted: ECL system, 5.0 mM $Ru(bpy)_3^{2+}$ in 50 mM pH 8.0 phosphate buffer; applied potential on Pt electrode, 1.17 V; separation voltage, 10 kV; running buffer, 10 mM pH 8.1 phosphate buffer; injection voltage, 10 kV; injection time, 10 s; THF concentration, 2% (v/v).

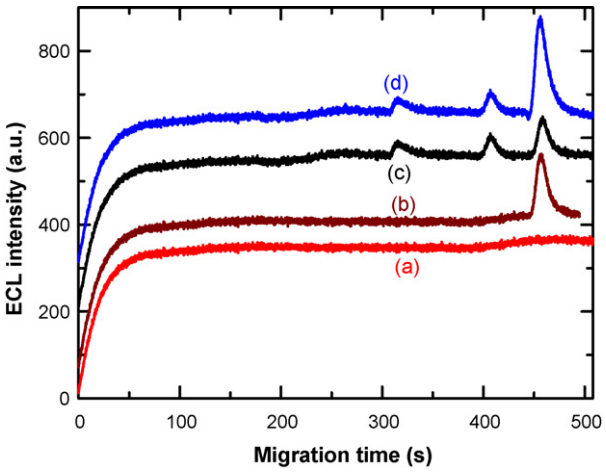

Fig. 10. Electropherograms of urine sample without bisoprolol (a), 3 μM bisoprolol standard solution (b), urine sample after 4 h administration (c), the mixture of urine sample after 4 h administration with standard bisoprolol solution (d). The peaks at around 470 s are from bisoprolol and other peaks are from impurities of samples. The experimental conditions are the same as in Fig. 9.

the range of 2.73–2.89%, indicating that the proposed method is efficient and sensitive.

4. Conclusions

In summary, capillary zone electrophoresis (CZE) coupled with end-column electrogenerated chemiluminescence (ECL) has been successfully utilized for the detection of bisoprolol in tablets and drugs. It is promising to determine all beta-blockers. The proposed CZE–ECL method shows a shorter migration time for bisoprolol than CZE–UV as well as HPLC. This method is rapid, simple and sensitive. Another advantage is that only aqueous solutions are required. This method is practical to be adopted as an official way to monitor bisoprolol in clinical and biochemical samples in future.

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