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Research Article

Simultaneous determination of ethamsylate, tramadol and lidocaine in human urine by capillary electrophoresis with electrochemiluminescence detection

Ethamsylate, tramadol and lidocaine, partly excreted by the kidney, are generally used as hemostatic, analgesic and local anesthetic in surgery. We developed a simple and sensitive method for their simultaneous monitoring in human urine based on CE coupled with electrochemiluminescence detection by end-column mode. Under optimized conditions the proposed method yielded linear ranges from 5.0×10^{-8} to 5.0×10^{-5}, 1.0×10^{-7} to 1.0×10^{-4} and 1.0×10^{-7} to 1.0×10^{-4} M with LODs of 8.0×10^{-9} M (36 amol), 1.6×10^{-8} M (72 amol) and 1.0×10^{-8} M (45 amol) (S/N = 3) for ethamsylate, tramadol and lidocaine, respectively. The RSD for their simultaneous detection at 1.0×10^{-6} M was 2.1, 2.8 and 3.2% (n = 7), respectively. For practical application an extraction step with ethyl acetate at pH 11 was performed to eliminate the influence of the sample ionic strength. The recoveries of ethamsylate, tramadol and lidocaine at different levels in human urine were between 87 and 95%. This method was used for simultaneous detection of ethamsylate, tramadol and lidocaine in clinic urine samples from two medicated patients. It was valuable in clinical and biochemical laboratories for monitoring these drugs for various purposes.

Keywords: Capillary electrophoresis / Electrochemiluminescence / Ethamsylate / Lidocaine / Tramadol DOI 10.1002/elps.200600135

1 Introduction

Ethamsylate, diethylammonium-2,5-dihydroxybenzenesulfonate (shown in Fig. 1), can be excreted by the kidney with original shape [1]. It is a systemic hemostatic and generally used for the prophylaxis and control of hemorrhages due to the rapture of small blood vessels in surgery and other clinical conditions [1–3]. Several methods such as potentiometry [4], biamperometry [5], square wave adsorptive stripping voltammetry [6], spectrophotometry [7] and chemiluminescence [8–10] have been developed for its detection. However, some methods either require carefulness to control measurement conditions or suffer from low selectivity.

In surgical procedures, ethamsylate is often used together with tramadol, a centrally acting analgesic, and lidocaine, one of the most extensively used local anesthetics, as hemostatic, analgetic or local anasthetic agent. The analgetic potency of tramadol is between weak opioid and morphine [11, 12]. It is mainly metabolized by liver and essentially excreted by the kidney [12, 13]. About 10% of lidocaine is excreted unchangingly in the urine [14]. The toxicity of lidocaine primarily affects the cardiovascular and central nervous systems [15]. The methods for determination of tramadol in biological samples involve HPLC and GC with the sample preparation of liquid–liquid extraction [16–18] and SPE [19–21]. These

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Abbreviations: ECL, electrochemiluminescence; **PMT**, photomultiplier tube; **$Ru(bpy)_3^{2+}$**, tris(2,2'-bipyridyl)ruthenium(II); **TPA**, tripropylamine

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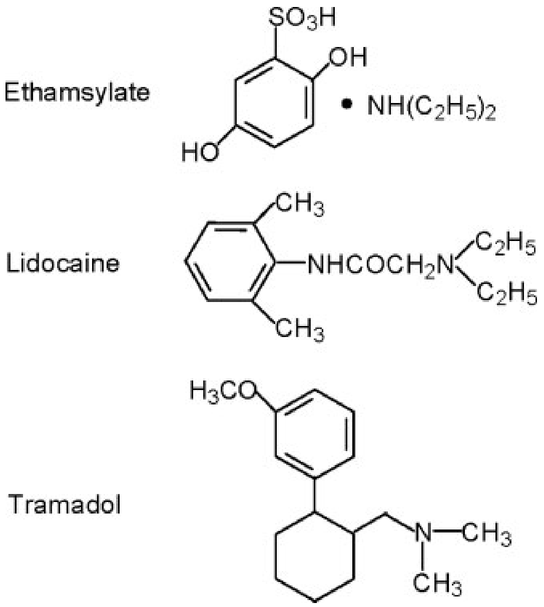

Figure 1. Formulas of ethamsylate, tramadol and lidocaine.

methods need a preconcentration step because of lack of a simple and highly sensitive detector. Recently, a solid-state electrochemiluminescence (ECL) detector for CE has been presented for the detection of lidocaine [22]. This method shows a linear range from 5.0×10^{-7} to 1.0×10^{-5} M. The analyses of lidocaine can also be done by GC with MS detection [23], HPLC with UV [24] or MS detection [25, 26]. Using an ECL detection two simple methods for sensitive detection of lidocaine have been proposed [27, 28]. The simultaneous determination of tramadol and lidocaine in urine can be performed by end-column CE [29]. However, to the best of our knowledge, the ECL determination of ethamsylate and the simultaneous determination of these three compounds have not been reported yet. A rapid method of their quantitative determination would benefit the study of their physiological function and the diagnosis of some diseases in clinical medicine.

CE has been developed as an efficient separation technique. The high efficiency and resolution potential, relatively short analytical time, low instrumental costs and small sample volume make CE an alternative to HPLC for the determination of drugs in biological fluids [30, 31] and the separation of metabolite mixtures [32, 33]. A series of methods based on CE with ECL detection of tris(2,2'-bipyridyl)ruthenium(II) ($Ru(bpy)_3^{2+}$), wherein a chemiluminescence reaction is initiated from reagents in the vicinity of the working electrode surface when potential is applied, have been developed for tertiary amines and their derivatives [22, 27–29, 34–37]. This work presents a novel method for sensitive determination of ethamsylate with ECL detection of $Ru(bpy)_3^{2+}$ system. We developed a rapid and highly selective CE-ECL method for the simultaneous determination of ethamsylate, tramadol and lidocaine in a small volume of urine sample. The application in monitoring ethamsylate, tramadol and lidocaine in the urine samples shows that this method is practical and valuable in clinical and biochemical laboratories for pharmaceuticals analysis and metabolism investigation. Thus, it could be very useful for carrying out simultaneous studies of the pharmacokinetics and pharmacodynamics of ethamsylate, tramadol and lidocaine.

2 Materials and methods

2.1 Materials

Ethamsylate, lidocaine hydrochloride and tramadol hydrochloride were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tris(2,2'-bipyridyl)ruthenium(II) chloride and tripropylamine (TPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade and used as received. All solutions were prepared with water purified in a Milli-Q System (Millipore, Bedford, MA). All standard solutions and phosphate buffers were prepared weekly, and filtered through 0.22 μm cellulose acetate filters (Shanghai Xinya Purification Material Factory) prior to injection.

2.2 Apparatus and procedures

A programmable high-voltage power supply (0–20 kV, Remax Electronic, Xi'an, China) was applied to perform the electrokinetic sample injection and electrophoretic separation. An uncoated fused-silica capillary with 50 cm length, 25 μm id and 360 μm od was used for separation (Yongnian Optical Fiber Factory, Hebei, China). Prior to use, the capillary was flushed with 0.1 M sodium hydroxide solution overnight. The electrochemical measurements in CE-ECL experiments were carried out on an MPI-A multifunctional electrochemical analytical system (Xi'an Remex Electronic and Technological) with a three-electrode system comprising a platinum wire as a counter, Ag/AgCl (3.0 M NaCl) as reference and a 500-μm platinum disk as working electrode. The ECL emission was detected with a Model BPCL Ultraweak Chemiluminescence Analyzer (Institute of Biophysics, Beijing) in a pulse mode, which was sensitive to photons with a wavelength range of 200–800 nm. The working electrode was adjusted and fixed by three screws from three different directions to align with the capillary under a microscope. The gap between working electrode and capillary was controlled at 70 ± 5 μm [38, 39]. The reference and counter-electrodes were inserted into the solution above both the capillary and the working electrode. The lower layer of cell was made of a piece of optical glass through which the photons were captured by a photomultiplier tube (PMT), which was biased at 800 V. Phosphate buffer (450 μL, 50 mM, pH 9.0) containing 5.0 mM $Ru(bpy)_3^{2+}$ was added to the cell for CE-ECL detection.

Electrophoresis in the capillary was driven by a high-voltage power supply (12 kV, 4 μA), which was applied at the injection end with the detection cell held at ground potential through the separation capillary guide. During the experiment, the separation voltage was applied at the injection end, and the reservoir in the ECL detection cell was held at ground potential. The detection potential was applied at the working electrode. In all experiments, sample introduction was accomplished by electrokinetic injection for 10 s at 10 kV (about 4.5 nL) [38]. Before use, the capillary was flushed with purified water and the running buffer (20 mM pH 9.0 phosphate buffer) for 15 min by means of a syringe. After each run the electrode was treated with a cyclic voltammetric scan in a potential range of −0.5 to 0 V at 100 mV/s for 2 min [29, 38], ascertaining to get better resolution and reproducibility. The resolution (R_s) was calculated according to Eq. (1) [36]:

$$R_s = 2(t_{r2} - t_{r1})/(w_1 + w_2) \qquad (1)$$

where t_{r1} and t_{r2} are the migration times of two adjacent peaks, and w_1 and w_2 are the baseline widths of the two adjacent peaks, respectively. After a stable baseline ECL signal was reached, electromigration injection was used for sample introduction, and the electropherogram was recorded. The sample concentrations were quantified by ECL peak intensities.

2.3 Sample preparation

The blank urine samples of healthy people collected from student volunteers in the laboratory were used as the matrices to spike ethamsylate, tramadol and lidocaine. The urine samples of patients were obtained from two men who were injected with 250 mg ethamsylate, 300 mg lidocaine and 100 mg tramadol before operation. The urine samples were collected after injection for 2, 4, 6 and 8 h, respectively, and stored at −20°C until experiment.

To eliminate the influence of sample ionic strength and obtain a clear electrophoretic profile, a modified Rurak's extraction procedure was performed before electrophoresis [40]. The urine samples (200 μL) or the spiked samples were firstly pipetted into clean 2.0 mL centrifugation tubes and alkalinized by adding 100 μL of saturated sodium carbonate solution, respectively. Ethyl acetate (1.0 mL) was then added to each sample. After the samples were vortex-mixed using a medium motion on a shaker for 30 min and centrifugated at 2000 rpm for 10 min, the top organic layers were separated and transferred into a clean set of centrifugation tubes. This procedure was repeated and the obtained organic layers were mixed. The mixtures were evaporated to dryness under a gentle stream of nitrogen at 35°C in a water bath. The residues were reconstituted with 200 μL of water and vortex mixed for 60 s. Finally, about 4.5 nL of the sample solutions were injected into the electrophoresis system by electromigration. The extraction efficiency at each analyte concentration was estimated by measuring the peak intensity of nonextracted standard solution compared with that of the corresponding spiked sample after extraction.

3 Results and discussion

3.1 Effect of applied potential on ECL response

As shown in Fig. 2, the effects of applied potential on the ECL responses of these compounds were investigated from +1.10 to +1.55 V (*vs.* Ag/AgCl). When the applied potential was less than +1.10 V, light emission was not observed since $Ru(bpy)_3^{2+}$ was not oxidized on the electrode. On the increasing applied potential from +1.10 V, the ECL intensity for ethamsylate, tramadol and lidocaine increased and then reached the maximum value. The ECL intensities for ethamsylate and tramadol showed the

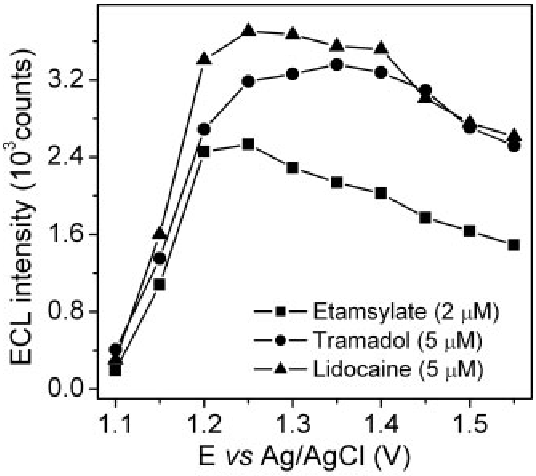

Figure 2. Effects of applied potential on ECL responses of 2.0 μM ethamsylate, 5.0 μM tramadol and 5.0 μM lidocaine. Injection, 10 kV for 10 s; running buffer, 20 mM phosphate buffer at pH 9.0; separation voltage, 12 kV; ECL cell, 5.0 mM $Ru(bpy)_3^{2+}$ + 50 mM phosphate buffer at pH 9.0; PMT voltage, −800 V.

maximum values at the applied potentials from +1.20 to +1.25 V and +1.25 to +1.40 V, respectively, while the applied potential for lidocaine was from +1.25 to +1.30 V. A more positive applied potential led to the decrease in their ECL responses. Thus, +1.25 V was selected as the optimum potential for the simultaneous determination of ethamsylate, tramadol and lidocaine. When the working electrode showed an obvious decrease in ECL signal, a cyclic voltammetric treatment of the electrode with a potential range of −0.5 to 0 V at 100 mV/s for 2 min could regenerate the electrode. With this treatment the electrode kept excellent stability of the ECL signal for two months without polishing or any other pretreatment step.

3.2 Effects of pH values of ECL cell and running buffer on ECL response

The pH values of both ECL detection solution and the running buffer showed obvious effects on the ECL response and resolution of these compounds. The ECL reaction of $Ru(bpy)_3^{2+}$ with alkylamine was a pH-dependent process with the maximum emission observed in slightly basic condition [41]. The slow kinetics associated with CL processes would produce a broad ECL reaction profile, thus leading to poor separation efficiency and low detection sensitivity [36, 42]. With the increasing pH value of the detection solution the ECL intensities for ethamsylate, tramadol and lidocaine increased and showed the maximum values in the pH range from 8.0 to 9.5, 8.0 to 9.0 and 8.0 to 9.5, respectively (Fig. 3). When the pH value was higher than these ranges, the ECL intensity decreased. Therefore, pH 9.0 was selected for their simultaneous detection.

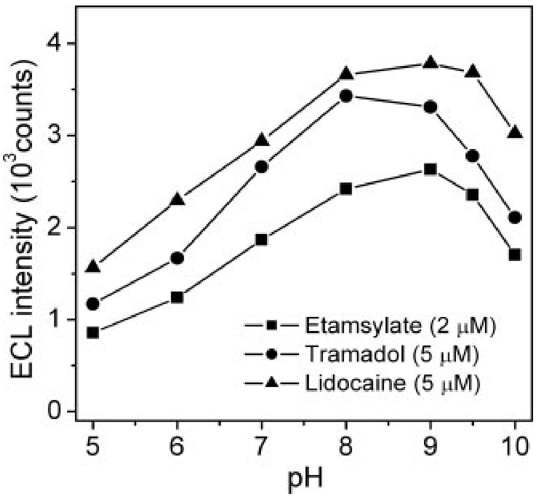

Figure 3. Effects of pH of ECL cell on ECL responses of 2.0 μM ethamsylate, 5.0 μM tramadol and 5.0 μM lidocaine. Detection potential, +1.25 V (*vs.* Ag/AgCl); other conditions as in Fig. 2.

The pH value of running buffer influenced mainly their resolution, which depended on the EOF. The EOF of the native capillary was determined by the degree of dissociation of the silanol groups and the ionic strength of the running buffer [43]. A high pH value led to high degree of dissociation and high EOF. Using TPA as an internal standard, the resolutions of ethamsylate and TPA (R_s1), TPA and tramadol (R_s2), tramadol and lidocaine (R_s3) were used to examine the effect of running buffer, respectively (Fig. 4). In an acidic environment, these compounds were cationic due to the protonation of tertiary amino groups [36, 44], and the dissociation degree of silanol groups was little, resulting in a low magnitude and small change of the EOF [45]. Thus these compounds showed both small changes and low values of resolution at low pH values. The R_s2 was even lower than 1.0 at the pH values lower than 7.2. With the increasing of pH, electroosmosis increased due to the dissociation of the silanol groups. Thus the resolution increased and reached gradually the maximum value. The dissociation of $-SO_3H$ group in ethamsylate made R_s1 reach first the maximum value of 3.1 at pH 9.0, at which the values of R_s2 and R_s3 were 2.2 and 2.3, respectively, indicating that the internal standard and three analytes were separated absolutely. A too high pH value of the running buffer resulted in the decrease of resolution due to the complete dissociation of the silanol groups and complete deprotonation of tertiary amino groups.

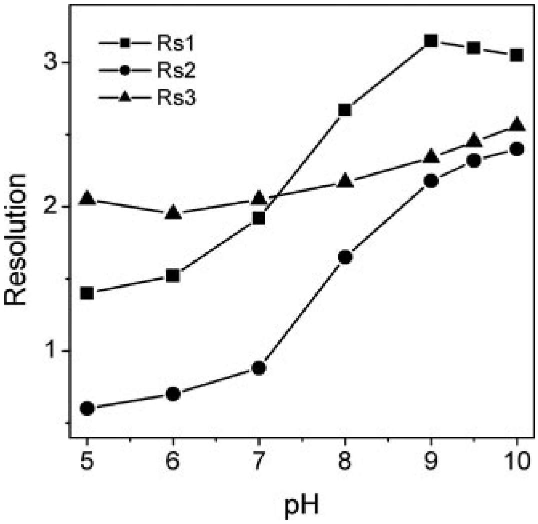

Figure 4. Effects of pH of running buffer on resolutions of ethamsylate/TPA (R_s1), TPA/tramadol (R_s2) and tramadol/lidocaine (R_s3) under other optimal conditions.

3.3 Effect of separation voltage on CE-ECL

The increase of separation voltage would increase the EOF and shorten the analytical time. However, the high voltage led to Joule heating and affected the separation of the analytes. The separation voltage was optimized in the range of 8–16 kV. Figure 5A shows the electropherograms of ethamsylate (a), TPA (b), tramadol (c) and lidocaine (d), obtained at various separation voltages. With increasing in separation voltage, EOF increased and retention time decreased (inset 1 in Fig. 5A). This observation was in agreement with the results reported previously [39, 46]. The short retention time made more analytes in the effluent arrive in the diffusion layer of the working electrode within a given period [47], which increased the reaction rate of $Ru(bpy)_3^{3+}$ with the analyte at the electrode surface [48] and produced better peak shape and higher ECL signal. On the other hand, the separation voltage also affected the separation efficiency. With the increasing in separation voltage, the strong flow of effluent from the capillary decreased the concentration of $Ru(bpy)_3^{3+}$ at the electrode surface, thereby reducing the efficiency of ECL reaction and the resolution. These two factors reached a compromise when the separation voltage ranged from 12 to 16 kV, so the ECL signal and resolution tended to a plateau (inset 2 in Fig. 5A). In order to obtain a good separation result and short analysis time, we selected 12 kV as the separation voltage in this work.

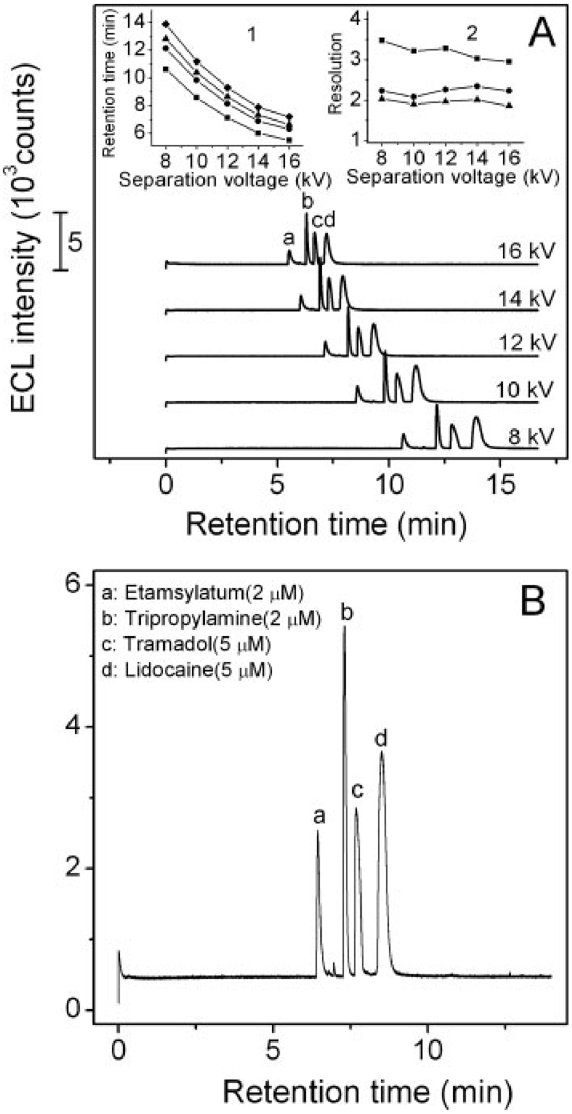

Figure 5. CE-ECL electropherograms for separation of 2.0 μM ethamsylate, 2.0 μM TPA, 5.0 μM tramadol and 5.0 μM lidocaine at different separation voltages (A) and typical electropherogram of 2.0 μM ethamsylate, 2.0 μM TPA, 5.0 μM tramadol and 5.0 μM lidocaine under optimal conditions (B). Inset in (A): effects of separation voltage on (1) retention times of ethamsylate (■), TPA (●), tramadol (▲) and lidocaine (◆) and (2) R_s1 (■), R_s2 (●) and R_s3 (▲).

3.4 Detection limit, linearity and reproducibility

Using the optimized experimental conditions such as applied potential, +1.25 V; running buffer, 20 mM pH 9.0 phosphate buffer; separation voltage, 12 kV; sample injection, 10 kV for 10 s; ECL detection cell, 5 mM $Ru(bpy)_3^{2+}$ in 50 mM pH 9.0 phosphate buffer, a typical electropherogram with a good separation of the three standard analytes and internal standard is shown in Fig. 5B. The retention time of the four compounds was within 9.5 min. The theoretical plate numbers (*N*) for ethamsylate, TPA, tramadol and lidocaine were 35 345, 99 762, 34 705 and 19 461 plates/m, respectively, calculated with $N = 5.54(t_R/W_{1/2})^2$, where t_R is the retention time of the analyte and $W_{1/2}$ is the width of the half peak. In the concentration range of 0.05–50.0, 0.1–100.0 and 0.1–100.0 μM for ethamsylate, tramadol and lidocaine, the ECL intensity was proportional to the concentration with the correlation coefficients more than 0.998, respectively. The LODs were determined to be 8.0×10^{-9} M (36 amol) for ethamsylate, 1.6×10^{-8} M (72 amol) for tramadol and 1.0×10^{-8} M (45 amol) for lidocaine at a S/N of 3.

Because electrokinetic injection mode was employed in this work, the sample ionic strength influenced the injection of samples. On the other hand, some organic compounds in urine would influence the ECL reaction. In order to obtain a clear electrophoretic sample profile, high detection sensitivity and good reproducibility, an extraction procedure was done to separate ions and some organic compounds in urine. During the extraction process, the pH value of the urine sample was adjusted to about 11.0 by adding 100 μL of saturated sodium carbonate solution into 200 μL of urine sample. At this pH, the three analytes existing in solution could be easily extracted into the organic phase. The extraction step, described in Section 2, was found to be the optimal condition to recover sufficient amounts of the three analytes expected in the urine sample, without coextracting endogenous interfering substances. To prevent analytes from decomposing, the evaporation process was endured at 35°C for 10 min. The residue was dried by nitrogen gas stream. The electropherograms for the blank and spiked blank urine samples after the extraction procedure are shown in Fig. 6. They showed the analytes could be separated from the noise of the blank urine, and the extraction step could exclude some interfering substances such as amino acids and peptides existing in urine [29]. Table 1 shows the recoveries of ethamsylate, tramadol and lidocaine spiked in blank urine sample. They were 87–92, 91–94 and 90–95%, respectively. The RSDs of ECL peak intensities were less than 8%.

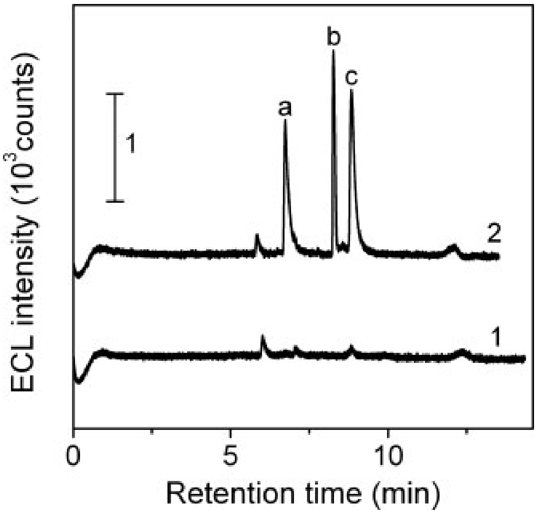

Figure 6. CE-ECL electropherograms of (1) blank urine sample and (2) the urine sample spiked with 0.8 μM ethamsylate (a), 3.0 μM tramadol (b) and 2.0 μM lidocaine (c) under optimal conditions.

3.5 Application

For the real analyses of urine samples from medicated patients, ethamsylate, tramadol and lidocaine were spiked in a blank urine matrix and extracted to obtain the working curve. The linear ranges for ethamsylate, tramadol and lidocaine were from 5.0×10^{-8} to 5.0×10^{-5}, 1.0×10^{-7} to 1.0×10^{-4} and 1.0×10^{-7} to 1.0×10^{-4} M with the correlation coefficients more than 0.998. The LODs were determined to be 8.0×10^{-9} M (36 amol), 1.6×10^{-8} M (72 amol) and 1.0×10^{-8} M (45 amol) for ethamsylate, tramadol and lidocaine at a S/N of 3, respectively. Although a compromise for simultaneous determination of three compounds was needed, they were lower than 1.0×10^{-6} M [27], 4.5×10^{-8} M [29] and 3.0×10^{-8} M for lidocaine [28] and 6.0×10^{-8} M for tramadol [29] with CE-ECL system. The LOD for lidocaine was also the same as that for lidocaine with solid-state ECL detector system [22]. Clearly, this method could be used to monitor sensitively ethamsylate, tramadol and lidocaine, and other pharmaceuticals containing tertiary amine groups in urine.

Figure 7A shows the electropherogram of an extracted urine sample from one patient after medicated for 2 h. The background of the electropherogram was considerably stable. To monitor the concentration changes of ethamsylate, tramadol and lidocaine levels in urine upon the injection time, the urine samples of two medicated patients were collected after injection for 2, 4, 6 and 8 h, respectively. The mean results are shown in Fig. 7B. Both tramadol and lidocaine reached their maximum concentrations after injection for 4 h, while ethamsylate reached the maximum level after injection for 6 h. The results for tramadol and lidocaine in urine changing with time were similar to those reported previously [29]. The result for ethamsylate could also be verified with the pharmacopoeia data [1].

4 Concluding remarks

This work reports a rapid, sensitive and highly selective CE method with ECL detection for the simultaneous determination of ethamsylate, tramadol and lidocaine in a small volume of urine. The optimal conditions are an applied potential of +1.25 V, a separation voltage of 12 kV, a running buffer of 20 mM at pH 9.0 with an ECL detection solution of 5 mM $Ru(bpy)_3^{2+}$ in 50 mM phosphate buffer at

Table 1. Recoveries of ethamsylate, tramadol and lidocaine in urine

Ethamsylate (*N* = 6)			Tramadol (*N* = 6)			Lidocaine (*N* = 6)		
Concentration	Recoveries (%)	RSD (%)	Concentration	Recoveries (%)	RSD (%)	Concentration	Recoveries (%)	RSD (%)
0.8	92	5.3	3.0	94	3.8	2.0	95	5.6
2.0	91	6.7	5.0	91	4.9	5.0	93	6.8
5.0	87	6.2	10	93	5.7	10	90	7.5

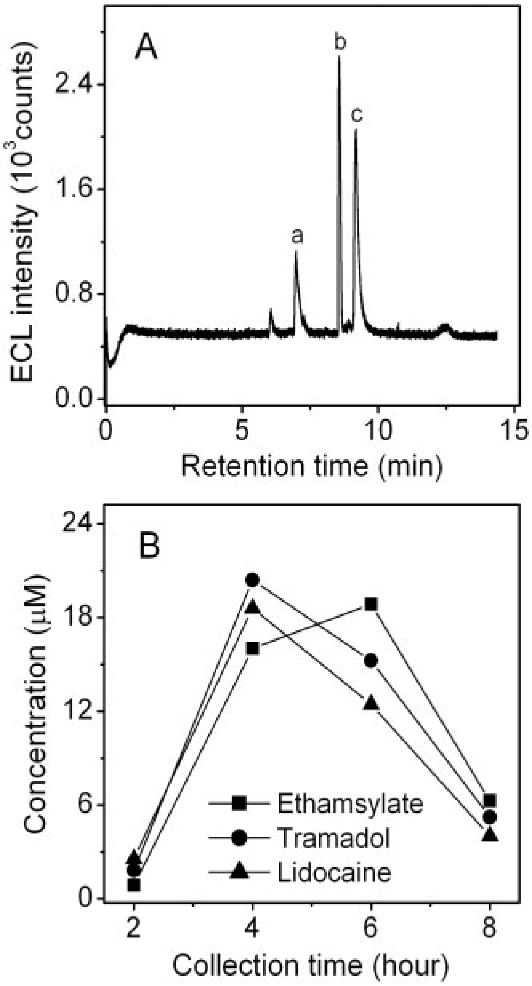

Figure 7. (A) CE-ECL electropherogram of patient urine after injection with ethamsylate (a), tramadol (b) and lidocaine (c); (B) changes in the concentrations of ethamsylate, tramadol and lidocaine in patient urine with collection time under optimal conditions.

pH 9.0. This method is practical and valuable in clinical and biochemical laboratories for pharmaceutical analysis and metabolism investigation. Since $Ru(bpy)_3^{3+}$-based ECL method is particularly suitable for those tertiary and secondary amines which are difficult to be detected by UV-visible absorption or fluorescence methods, the preliminary results promise that the CE-ECL system is a convenient and sensitive approach and can be used for detecting medicines containing tertiary or secondary amino group.

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