Feature Article

Simultaneous Determination of Tramadol and Lidocaine in Urine by End-column Capillary Electrophoresis with Electrochemiluminescence Detection

Weidong Cao, *Jifeng Liu*, *Haibo Qiu*, *Xiurong Yang*,* *Erkang Wang**

State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China; e-mail: ekwang@ns.ciac.jl.cn

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Abstract
Tramadol and lidocaine, used as analgesic and local anesthetic in surgery, are partly excreted by kidney. For the first time, we developed a simple and sensitive method, based on capillary electrophoresis with electrochemiluminescence (ECL) detection by end column mode without joint to monitor tramadol and lidocaine in urine. To eliminate the influence of ionic strength of urine sample, analytes were extracted by ether. Tripropylamine (TPA) was used as internal standard. The recoveries of tramadol and lidocaine were between 94% and 97% at different levels. The method exhibited the linear range for the tramadol and lidocaine from 1.0×10^{-7} to 1.0×10^{-4} mol/L with correlation efficient of 0.998. The relative standard deviation (RSD) was 2.9% and 2.7% ($n = 8$) for tramadol and lidocaine, respectively. The limit of detection (LOD) was 6.0×10^{-8} mol/L and 4.5×10^{-8} mol/L (S/N = 3) for tramadol and lidocaine, respectively. The application for detecting tramadol and lidocaine in urine of patients showed that the method was valuable in clinical and biochemical laboratories for detecting tramadol, lidocaine and other tertiary amine pharmaceuticals for various purpose, such as metabolism investigation.

Keywords: Capillary electrophoresis, Electrochemiluminescence, Tramadol, Lidocaine

1. Introduction

Tramadol[1-(e)-(*m*-methoxyphenyl)-2-(e)-(dimethylaminomethyl)-cyclohexane-1 -(a)-ol] is a centrally acting analgesic that possesses an analgetic action with a potency ranging between weak opioids and morphine [1, 2]. Clinical studies have shown that tramadol does not have a pronounced opioid side-effect profile. Little respiratory depression and no analgetic tolerance after repeated administration were observed with tramadol [3]. Tramadol may exert part of its analgesic effect through activity on the monoaminergic [4]. About 85% of a dose of tramadol is metabolized by liver and essentially excreted by the kidney [2, 5]. While lidocaine, an amide synthesized from cocaine, is one of the most extensively used local anesthetics. It is administered parenterally for ventricular arrhythmia, subcutaneously for minor surgical procedures. Less than 10% of lidocaine is excreted unchanged in the urine [6]. Lidocaine toxicity primarily affects the cardiovascular and central nervous systems. Toxicity is enhanced in patients prone to decreased lidocaine clearance including those with congestive heart failure and liver cirrhosis, and in patients taking certain medications such as β-blockers and cimetidine [7]. In the surgical procedures, tramadol and lidocaine are often used for analgetic and local anesthetic, respectively.

Analytical methods for detecting tramadol and lidocaine are gas chromatography [8, 9] with nitrogen-phosphorus detection or mass spectrometry [10, 11], high-performance liquid chromatography (HPLC) with UV, fluorimetric detection or electrochemical detection [12, 13, 14]. But these methods need preconcentration before determination because of lacking high sensitive detector.

Capillary electrophoresis (CE) has been developed as an efficient separation technique. The high efficiency and resolution potential, relatively short analysis time, low instrumental cost, and small sample volume make CE an alternative to HPLC. By this method, tramadol and lidocaine are detected with UV [15, 16], MS [17] or LIF [18]. Whereas, either low sensitivity as UV, or complicated equipment and high price of detector as LIF and MS limit related practical application.

ECL of $Ru(bpy)_3^{2+}$, a high sensitive and selective analytical strategy wherein a chemiluminescence reaction is initiated from reagents in the vicinity of the working electrode surface when potential is applied, has been widely applied [19]. Because of intrinsic characters of ECL of $Ru(bpy)_3^{2+}$, it shows especially high sensitivity for tertiary amines [20, 21], and it is used to detect amino acid, peptides and β-blockers [22, 23, 24, 34, 35, 36] for CE.

For the first time, we designed a simple and high sensitive CE-ECL system without the joint to couple ECL detector with CE directly to detect tramadol and lidocaine. By adopting 25 μm i.d. capillary as separation column and low ionic strength solution (10 mM phosphate) as running buffer, the influence of current of electrophoresis on ECL is eliminated. In addition, by using large Pt disk electrode

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(300 μm diameter) as working electrode, the amount of oxidized $Ru(bpy)_3^{2+}$ is enough to ascertain sufficient $Ru(bpy)_3^{3+}$ generated at the end of the capillary. Using TPA as internal standard and double extraction steps by ether, the recovery of tramadol and lidocaine at three different concentration levels spiked in blank urine matrix is between 94 – 96% and 93 – 97%, respectively. The LOD for tramadol and lidocaine reached to 6.0×10^{-8} mol/L and 4.5×10^{-8} mol/L, respectively. The application for detecting tramadol and lidocaine in the urine samples of patients implied this method was practical and valuable in clinical and biochemical laboratories for the determination for tramadol and lidocaine for pharmaceuticals analysis, metabolism investigation and function.

2. Experimental

2.1. Reagents

All reagents used were commercially available and of analytical grade. TPA was purchased from Sigma and used as received. The lidocaine and the tramadol hydrochloride were obtained from Beijing Yongkang Pharmaceutical Factory and Grunenthal Pharmaceutical Company respectively. Tris(2,2′-bipyridyl) ruthenium(II) chloride were purchased from Aldrich. All solutions were prepared with water purified in a Milli-Q System (Millipore, Bedford, MA). The stock solutions were stored in the refrigerator (4 °C). The ether reagent was purified by 0.1 mol/L HCl before use. All standard solutions and phosphate buffers were prepared daily and filtered through 0.22 μm membrane prior to injection.

2.2. Apparatus

Electrophoresis in the capillary was driven by a high-voltage power supply (Spellman CZE 1000R, Plainview, NY). A 50 cm length of 25 μm i.d. and 320 μm o.d. uncoated fused-silica capillary was used (Yongnian Optical Fiber Factory, Hebei, China). The capillary was flushed with 0.1 mol/L sodium hydroxide solution overnight before use. The electrochemical measurement coupled with ECL experiments were carried out with a CH Instruments Model 800 Voltammetric Analyzer (USA). A three-electrode configuration was employed with a Pt wire as a counter electrode, Ag/AgCl as a reference electrode, and 300 μm in diameter disk Pt as a working electrode. The ECL emission was detected with a Model BPCL Ultra Weak Luminescence Analyzer (Institute of Biophysics, Chinese Academy of Sciences). To achieve high ratio of S/N, the photomultiplier tube (PMT) used in the BPCL ultra-weak luminescence analyzer was operated in pulse mode. It is sensitive to photons with wavelengths ranging from 400 to 800 nm.

2.3. Preparation of the Standards

TPA was used as internal standard. 1.0 mmol/L TPA stock solution was prepared with deionized water and stored at 4 °C. Further dilutions of the stock solutions with deionized water to obtain final internal standard concentration were made daily. Stock solutions of tramadol and lidocaine containing 1.0 mmol/L were weekly prepared in deionized water and stored at 4 °C. Standard solutions of tramadol and lidocaine were prepared by appropriate dilution with deionized water of the stock solution.

2.4. ECL Cell Design

The construction of ECL cell is shown in Figure 1. The cell body was made of polymethyl methacrylate material. To avoid capillary absorbing photon, a 3.0 mm long polyimide coating at the end of separation capillary was removed. Then the bare capillary end was cleaned by water in ultrasonic cleaner. A stainless steel tube (4.0 cm) was bundled near the end of separation capillary (about 1.5 cm), which was inserted in the solution about 1 cm. The ground electrode of the separation voltage was attached to the stainless steel tube. Due to adopting 25 μm i.d. capillary, electrophoresis current was reduced greatly and mainly flowed into ground electrode of separation voltage. Alignment of the working electrode with the end of capillary was quite important in the experiment. To accomplish alignment exactly, the working electrode was adjusted and fixed by three screws from three different directions to align with the capillary under the microscope. The gap between

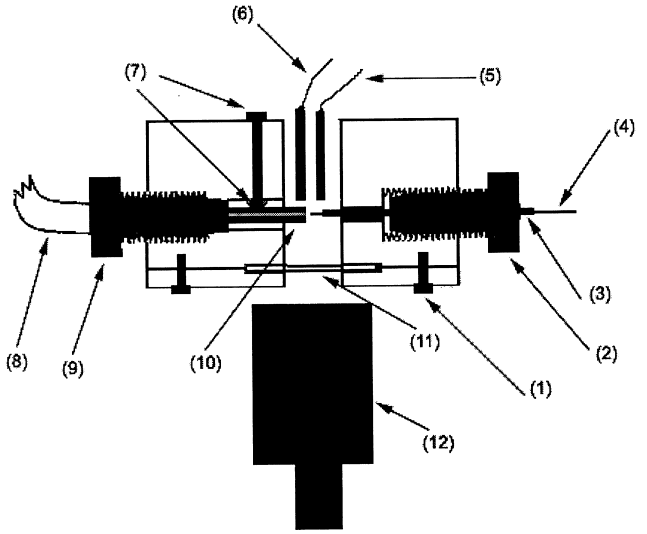

Fig. 1. Schematic diagram of the ECL cell coupled with separation capillary by end column mode. 1) Stainless steel holding screw, 2) PVC capillary holder, 3) stainless steel tube, 4) separation capillary, 5) reference electrode, 6) counter electrode, 7) Nylon screws for alignment, 8) working electrode cable 9) PVC electrode holder, 10) working electrode, 11) optic glass window, 12) PMT.

working electrode and capillary was controlled at 70 ± 5 μm. The lower layer of cell was made of a piece of optic glass through which the photons were captured by PMT. The reference electrode and the counter electrode were inserted into solution above the capillary and the working electrode to avoid blocking photon into PMT. The distance between the reference and the working electrode was kept 0.5 mm.

2.5. The Activation of the Working Electrode

The working electrode was made by sealing a 300 μm diameter Pt wire in a polystyrene tube to form disk electrode. Before use, the working electrode surface was polished with 0.3 μm α-alumina powder and washed by water in a ultrasonic cleaner. Because the applied potential of the ECL is as high as 1.2 V (vs. Ag/AgCl), it makes the surface of the electrode easily oxidized to form oxidization layer resulting in decreasing the ECL intensity [25]. Thus, to assure the reproducible electrochemical and ECL behavior, after each run a reactivated process was done by means of cyclic voltammetric scanning for 2 min between − 0.5 V and 0.0 V (vs. Ag/AgCl) at a scan rate of 100 mV/s to eliminate the oxidization layer on the electrode. By this electrochemical treatment, the experiments showed that the working electrode could work normally and kept stable at least two months without polishing again.

2.6. CE Separation and Detection Conditions

Electrokinetic injection was performed by applying 10 kV of voltage (positive at inlet end) for 10 s. Electrophoresis was carried out at 15 kV for 8 min. A 10.0 mmol/L phosphate buffer adjusted to pH 9.0 with 0.1 mol/L NaOH was used as running buffer. The capillary was regenerated by flushing with 0.1 M sodium hydroxide for 1 min after each run followed by a 2 min water rinse and 2 min buffer rinse in order to ascertain to get better resolution and reproducibility. About 300 μL ECL solution containing 50 mM phosphate buffer and 5 mM tris(2,2′-bipyridyl) ruthenium(II) was filled in the ECL detection cell.

2.7. Sample Preparation

The blank urine samples of healthy persons collected from student volunteers in the laboratory were used as matrix spiking TPA, tramadol and lidocaine. The urine samples of patients were obtained from two women who had been injected lidocaine and tramadol during operation. The urine samples were collected after surgery 2 hours, 4 hours and 6 hours, respectively. The sample, stored at − 20 °C before experiment, was treated and detected within a day. To eliminate the influence of ionic strength in sample and obtain clear electrophoretic profile a series of extraction procedures were done before electrophoresis. First, adding 500 μL ethyl ether and 50 μL 0.1 mol/L sodium hydroxide into a 500 μL aliquot of urine sample in a 1.5 mL Eppendorf tube. Then, the sample was vortex mixed for 10 min and centrifuged at 2000 rpm for 5 min. After that the organic layer was removed into another clean Eppendorf tube. Finally, the organic layer was evaporated at 40 °C and dried under a gentle steam of nitrogen. The dry residue was dissolved in 500 μL of water by vortex mixing during 5 min.

3. Results and Discussion

The previous work has shown that by using end-column mode, the separation voltage influences the electrochemical detection system. In order to reduce the influence of separation voltage, on-column fracture is designed to separate current of electrophoresis from electrochemical detection system [26, 27, 28, 29]. However, the decoupler makes the CE-EC system more complicated. In addition, the on-column fractures easily causing peak broadening [37]. Alternatively, by employing 25 μm i.d. narrow capillary as separation column, the remaining potential drop within the detector becomes too small enough to interfere with the electrochemical detection [38]. By this strategy the on-column fracture is unnecessary. Thus, the CE-EC system becomes easier to construct. Accordingly, we adopted this strategy to couple CE with ECL detection by end column mode. ECL detection cell was filled with 50 mmol/L phosphate supporting electrolyte and $Ru(bpy)_3^{2+}$. The distance between end of capillary and working electrode was kept to 70 μm. As shown in Figure 2, the applied potential was investigated towards tramadol and lidocaine by changing from 0.2 V to 1.4 V (vs. Ag/AgCl). When the applied potential was less than 1.0 V, light emission was not observed since $Ru(bpy)_3^{2+}$ was not oxidized on the electrode. While above 1.0 V, ECL intensity for lidocaine and tramadol increased with the applied potential. Lidocaine is more sensitive than tramadol. The ECL intensity for tramadol showed constant sensitive within the range 1.10 to 1.25 V. For lidocaine the intensity reached to maximum value at 1.2 V. When the potential moved to 1.3 V, the light intensity for two compounds decreased slightly. Clearly, the optimum potential for ECL is 1.2 V, which is in accordance with the redox potential of $Ru(bpy)_3^{2+}$. Therefore, we conclude that the applied potential *is* not shifted significantly under the influence of separation voltage.

Generation of a large amount of $Ru(bpy)_3^{3+}$ at the end of capillary is important for achieving high sensitivity. Employing comparatively large working electrode is a simple way to produce more $Ru(bpy)_3^{3+}$ at the end of capillary. Therefore, we used 300 μm diameter Pt as the working electrode.

Different groups [32, 33] have studied the difference of ECL dependence on pH for tertiary amines. The optimum pH value for tertiary amine is about 8 – 9. The effect of pH value of both the ECL solution and the running buffer on ECL intensity was investigated from pH 4 to 10. We found the ECL intensity was less depended on the pH value of running buffer, but mainly on the pH of ECL solution. Altering the pH value of running buffer did not bring much

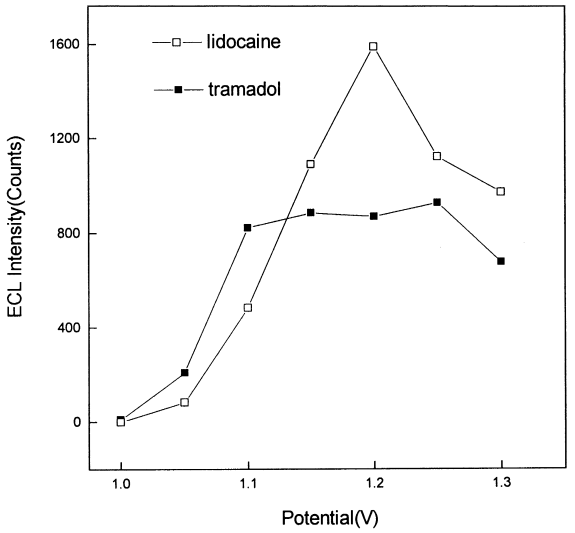

Fig. 2. Effect of the applied potential on ECL for tramadol (1.0 μmol/L) and lidocaine (5.0 μmol/L).

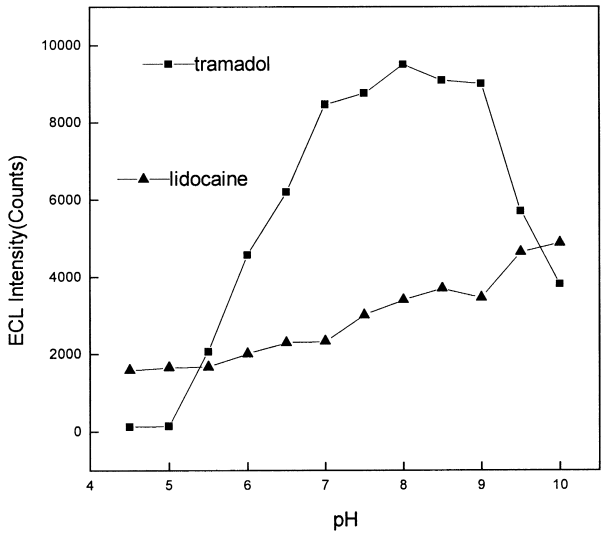

Fig. 3. Effects of pH on ECL intensity for lidocaine (1.0 μmol/L) and lidocaine (3.0 μmol/L).

vibration of light emission. It can be explained that only a small amount of running buffer flowing into the ECL cell when adopting 25 μm i.d. capillary as separation column, and the ECL solution has enough buffer capacity. Therefore, the pH value of the ECL solution did not change. While changing the pH value of ECL solution, the ECL intensity changed greatly. As illustrated in Figure 3, the ECL intensity for tramadol reached the highest at pH 8.0. When pH was over 9, the ECL intensity decreased slightly. However, the ECL intensities for lidocaine increased significantly over pH 9–10. Therefore, pH 9.0 was selected for detecting tramadol and lidocaine. However, the pH value of running buffer also influences the resolution of tramadol, lidocaine and internal standard. As shown in Figure 4, when pH was 6.0, the resolution of TPA/tramadol

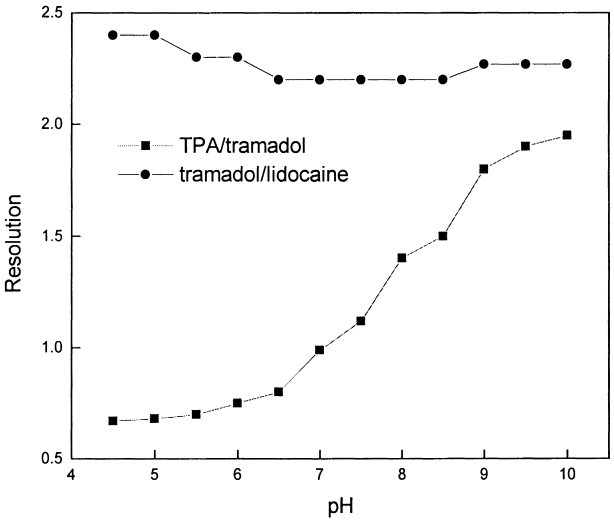

Fig. 4. Effects of pH on the resolutions of TPA/tramadol and tramadol/lidocaine.

and tramadol/lidocaine was lower than 1.0. It can be explained that the increase of pH resulted in larger electro-osmosis, which brought the rising of resolution (R_S). When pH value of running buffer was 9.0 the resolution for TPA/tramadol and tramadol/lidocaine reached to 1.7 and 2.1, respectively. The internal standard and two analytes were separated absolutely.

In order to eliminate the influence of variation in the electrokinetic injection and extraction process, TPA was used as an internal standard for the quantitative determination, and was added into urine sample before extraction. Because electrokinetic injection mode is employed in this work, the ionic strength of sample matrix will influence the injection of samples. The larger difference of ionic strength between running buffer and sample is advantageous for enhancing the on-column stacking efficiency of analytes. On the other hand, some organic compounds in urine may influence the ECL reaction. Therefore, in order to obtain a clear electrophoretic sample profile, high detection sensitivity and good reproducibility, the extraction procedure was done to separate ions and some organic compounds in urine.

During extraction process, the pH value of urine sample was adjusted to about 12.0 by adding 50 μL 0.1 mol/L sodium hydroxide into 500 μL urine sample. Under this pH condition the two analytes and internal standard existed in solution were easily extracted into organic phase. The extraction step, described in the experimental section, was found to be the optimal condition to recover sufficient amounts of the two analytes and internal standard expected in urine sample, without co-extracting endogenous interfering substances. To prevent analytes from decomposing, the process of evaporating was endured at 40 °C for 10 minutes. The residue was dried by nitrogen gas stream. Table 1 shows the recoveries of tramadol and lidocaine spiked in blank urine sample. The recoveries of tramadol and lidocaine in urine sample were between 94–96% and 95–97%, respectively.

Table 1. The recoveries of tramadol and lidocaine in urine sample. TPA was used as internal standard.

Tramadol (N = 6)			*Lidocaine (N = 6)*		
Concentration	*Recoveries (%)*	*RSD (%)*	*Concentration*	*Recoveries (%)*	*RSD (%)*
0.5	96	6.3	0.5	97	5.4
2	94	8.6	2	93	6.2
5	94	6.2	10	96	6.6

Table 2. The concentration dependent range of tramadol and lidocaine. TPA was used as internal standard.

Concentration	$Y = A + B \times X$					
Range (μM)	*Tramadol*			*Lidocaine*		
	A	*B*	*R*	*A*	*B*	*R*
0.2 – 10.0	0.02	1.36	0.998	0.03	2.3	0.999
20.0 – 100.0	0.34	0.0832	0.998	0.221	0.0863	0.998

Under the following conditions for detecting tramadol and lidocaine: applied potential 1.2 V; ECL solution containing 50.0 mmol/L phosphate (pH 9.0) and 5.0 mmol/L $Ru(bpy)_3^{2+}$; 10.0 mmol/L phosphate (pH 9.0) as running buffer; the precision of the method is assessed using eight replicate injections of a 1.0 μmol/L solution of tramadol and lidocaine. The internal standard TPA is 0.1 μmol/L. The relative standard deviation (RSD) of the peak height of the components are 2.9% and 2.7% for tramadol and lidocaine respectively. The calibration plot obtained by replicate analysis ($n = 3$) of series of analytes concentrations corresponding to 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100.0 μmol/L are subjected to linear regression analysis as shown in Table 2. 0.1 μmol/L TPA and 1.0 μmol/L TPA are used as internal standard for 0.2 to 10.0 μmol/L and 10.0 to 100.0 μmol/L analytes respectively. In the range of 0.1 to 10.0 μmol/L, the linear regression analysis: $y = 0.02 + 1.36x$ and $y = 0.03 + 2.30x$ for tramadol and lidocaine respectively, where y is the peak height of TPA/peak height of tramadol or lidocaine, x is the concentration of analytes in μmol/L. The LOD with a signal to noise ratio of 3 was determined to be 6.0×10^{-8} mol/L and 4.5×10^{-8} mol/L for tramadol and lidocaine, respectively.

The two patients were administrated lidocaine and tramadol during operation. To detect the concentration of tramadol and lidocaine in urine, the urine samples were collected about two hours after operation. The electro-

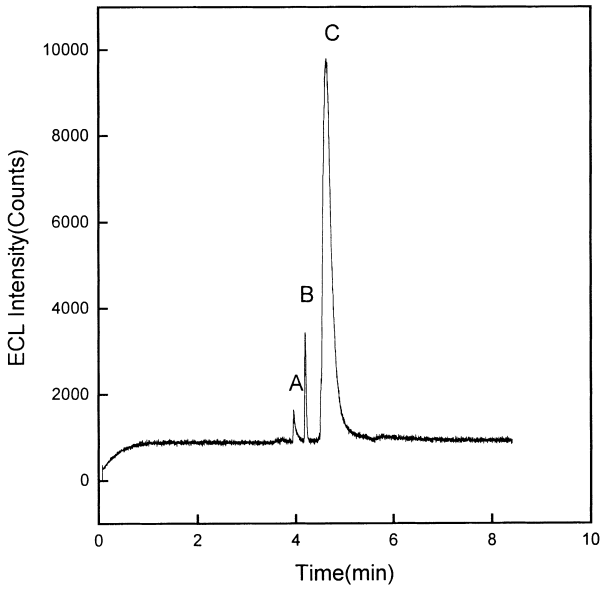

Fig. 5. Typical electrophoretic profile of tramadol (B) and lidocaine (C) in patient urine. TPA (A) was used as internal standard (0.1 μmol/L). Electrophoresis condition: 10 mmol/L phosphate buffer (pH 9.0) was used as running buffer; separation capillary 50 cm length, 25 μm i.d.; separation voltage 15 kV; sample injection 10 s at 10 kV. Detection condition: applied potential 1.20 V (vs. Ag/AgCl); $Ru(bpy)_3^{2+}$ 5 mmol/L; phosphate buffer (pH 9.0) 50 mmol/L.

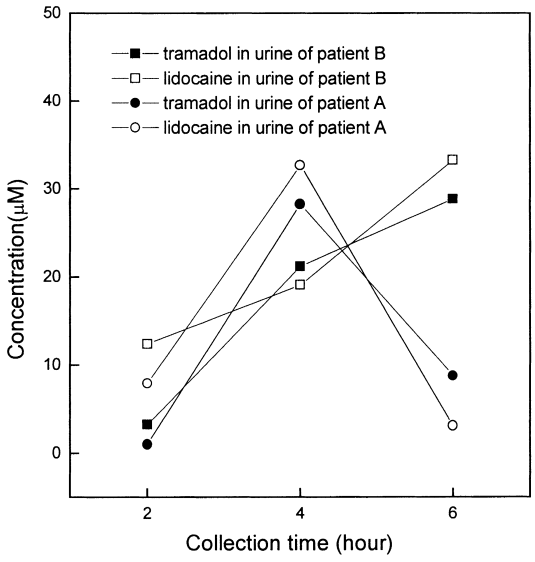

Fig. 6. Changes of the concentration of tramadol and lidocaine in the urine of two patients with time.

phoresis profile of one of patient urine sample is shown in Figure 5. The background line of electrophoresis is considerably stable. Through the extraction step, the analytes were selectively extracted and some interfering substances such as amino acid and peptides existing in urine were separated. To monitor the change of tramadol and lidocaine levels in urine with time, the second and third urine samples were collected about four hours and six hours, respectively, after operation. The results for tramadol and lidocaine in urine changing with time are shown in Figure 6. For patient A, the concentrations of the two pharmaceuticals in urine reached to the highest after 4 hours. Whereas, for patient B, the concentrations of pharmaceuticals in urine still kept high. We observed that the amount of urine patient A excreted was larger than that for patient B.

4. Conclusion

By adopting a 25 μm i. d. capillary serving for separation and choosing proper buffer the influence of separation voltage on ECL could be eliminated without making a fracture on column. The detection for tramadol and lidocaine in urine indicates that the simplified CE-ECL device provides high sensitivity, wide linear range, satisfying linear relationship and excellent reproducibility for detecting medicine containing tertiary amino group. This new strategy also provides a simple and practicable way to detect amino acids, peptides and protein.

5. Acknowledgement

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