Simultaneous Determination of Tetracaine, Proline, and Enoxacin in Human Urine by CE with ECL Detection

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Abstract

A simple, fast, and sensitive capillary electrophoresis method was developed for the simultaneous determination of tetracaine, proline, and enoxacin in human urine with electrochemiluminescence (ECL) detection. The effects of experimental conditions including the mode of applied voltage signal, the potential of working electrode, pH value, the flow rate of carrier of solution, the concentration of $Ru(bpy)_3^{2+}$, and the ECL intensity of the drugs were investigated in detail. Parameters related to the separation and detection were investigated and optimized. Under optimized conditions, the proposed method displayed a linear range from 0.4 to 100 µg/mL for tetracaine, 0.2 to 80 µg/mL for proline, and 0.1 to 100 µg/mL for enoxacin. Their limits of detection were 0.08, 0.06, and 0.02 µg/mL, respectively. A baseline separation for tetracaine, proline, and enoxacin was achieved within 10 min. Developed method was successfully applied to determine tetracaine, proline, and enoxacin in human urine.

Introduction

Enoxacin is used in the treatment of systemic infections including urinary tract, respiratory gastro-intestinal, and skin infections (1). The mechanism of effect is based on the inhibition of the DNA-gyrase of bacteria and RNA and protein synthesis. Tetracaine hydrochloride was used in hospitals as a kind of analgesic or local anesthetic, which induced bilayer interdigitation of phosphatidylcholines containing various acyl chain lengths (2). The first case of methemoglobinemia associated with tetracaine lozenge use was described (3). The tetracaine excreted unmetabolized in urine. It is significant to estimate the concentrations of tetracaine in urine after possible inadvertent intra-arterial injection or intrathecal injection, in the case when the administration of an excessive dose of local anesthetic causes systemic toxic reactions, and to guide clinical use of local anesthetics. Because tetracaine was used as anesthetics agent before an operation and enoxacin was used as bactericide and analgesic agent after the operation, fast analysis of enoxacin and tetracaine is of clinical importance for understanding the patient's medical process. Otherwise, proline as a predominant amino acid in the grape berry was commonly found in biological fluids in free-, peptide-, and protein-form, and the contents of proline are in association with various diseases such as bone diseases, tumors, and chronicuremia (4). For clinical study, it is required to establish a fast and sensitive analytical method for detection of proline in biological fluids.

Several methods have been reported for the determination of enoxacin by spectrophotometric (5), spectrofluorometry (6), liquid chromatography (7–9), chemiluminescence (CL) (10) and capillary electrophoresis (CE) (11), and electrogenerated chemiluminescence (ECL) (12). There have also been reports of quantification of the tetracaine by spectrophotometry (13), liquid chromatography (14,15), CL (16,17) and CE (18), as well as for the determination of proline by spectrophotometry (19), liquid chromatography (20,21), CL (22), and CE (23).

Tris(2,2'-bipyridine)ruthenium(II) [$Ru(bpy)_3^{2+}$] is one of the most extensively studied and used ECL compounds owing to its superior properties, which include high sensitivity and stability under moderate conditions in aqueous solution. Noffsinger and Danielson first reported the CL of $Ru(bpy)_3^{2+}$ with aliphatic amines (24). The feasibility of using $Ru(bpy)_3^{3+}$ CL reaction for the detection of amino acids, peptides, and proteins was studied (25). Nieman and co-works reported several liquid chromatographic methods with ECL detection using $Ru(bpy)_3^{2+}$ for the determination of dansyl amino acids (26), oxalate in urine and plasma (27), dansyl amino acids and oxalate (28), erythromycin in urine and plasma (29), glyphosate, and related compounds (30). This method was also used for the determination of oxalate and proline (31).

CE has increasingly become an efficient separation technique. Separations with several hundred thousand theoretical plates have already been achieved even with simple capillary zone electrophoresis in its early day (32). The high efficiency, powerful resolution, fast separation, and low instrumental cost as well as low consumption of samples and reagents are the main advantages of CE over high-performance liquid chromatography (HPLC). Wang and co-works reported several CE methods with ECL detection using $Ru(bpy)_3^{2+}$ for the determination of procyclidine in human urine (33), diphenhydramine in rabbit plasma

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and urine samples (34), lincomycin in the urine (35) as well as lidocaine and ofloxacin (36).

The CE coupling with ECL detection was reviewed (37). ECL detection, in comparison with other modes, offers lower background noise, higher detection sensitivity, and requires simple and inexpensive instrumentation. The CE coupling with ECL of $Ru(bpy)_3^{2+}$ has been applied for the determination of proline with the detection limit of 0.88 µg/mL (38), 0.22 µg/mL (39), and 0.43 µg/mL (40). To our knowledge, there is no report for the determination of tetracaine and/or enorxacin by capillary electrophoresis with $Ru(bpy)_3^{2+}$ ECL. The aim of this study was to develop the efficient method for simultaneous determination of tetracaine, proline, and enoxacin by CE with ECL detection with $Ru(bpy)_3^{2+}$. Both separation conditions and electrochemical reaction parameters were optimized. The proposed method was applied for simultaneous determination of tetracaine, proline, and enoxacin in urine samples with satisfactory results.

Experimental

Reagents and Chemicals

Proline was obtained from Sigma (St. Louis, MO). Tris(2,2'-bipydidyl)ruthenium(II) chloride hexahydrate (TBR) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Enoxacin and tetracaine were obtained from Institute of Medical Biotechnology (Beijing, China). Phosphate buffer both used in the detection cell and as electrophoresis running buffer was prepared by equimolar amount of disodium hydrogenphosphate and sodium dihydrogenphosphate. The appropriate pH of the buffer was adjusted with orthophosphoric acid or sodium hydroxide. Human urine was provided by healthy volunteers. All chemicals including phosphate, sodium hydroxide were of analytical-grade. The double-distilled water was prepared using XGJ-30 water purification system (Yongcheng Company, Beijing, China). Prior to CE analysis, the drug solution and buffer were filtered through a 0.22-µm cellulose acetate membrane before use.

Standard solution and sample preparation

The stock solution of 0.5 mg/mL tetracaine, proline, and enoxacin was prepared by dissolving 25.00 mg, 25.00 mg, and 27.442 mg, respectively, in 50 mL water and stored at 4ºC in a refrigerator. Before use, the stock solution was diluted with water to prepare standard solutions with a series of concentrations for the construction of calibration curve and the study of reproducibility and recovery. The blank urine was provided by student volunteers in the laboratory.

A 1-mL volume of urine sample was deproteinized by adding 1.0 mL 10% trichloroacetic acid (CCl_3COOH) in a centrifuge tube, which was then centrifuged for 15 min at 4000 rpm. The centrifugate was required for urine samples.

Apparatus

All experiments were carried out on a computer controlled CE–ECL system (Xi'an Remax Electronics, Xi'an, China), including a high voltage power supply for electrophoretic separation and electrokinetic injection, a potential control system, a CL detector, and a data processor. A three-electrode configuration was used in the detection system consisting of a 500-µm Pt disk as a working electrode, Ag/AgCl as a reference electrode, and Pt wire as a counter electrode. The end-column detection was employed by using a wall-jet configuration. The distance between working electrode and capillary was controlled at 70 µm in order to obtain higher ECL intensity and theoretical plate number (41). The reference and counter electrodes were inserted into the previous solutions as well as both the capillary and working electrode.

Separations were performed in 35-cm long fused-silica capillaries (Yongnian Optical Fabric Factory, Hebei, China) with a 25-µm i.d. and 360-µm o.d. The capillary was filled with 0.1 M sodium hydroxide overnight.

Electrode preparation

The working electrode was polished with pieces of sandpaper (0.3 µm and 0.05 µm-alumina powder in turn) until a mirror-smooth surface appeared. It was then sonicated for 2 min and rinsed with double-distilled water to make it sufficiently clean before using. Once fouling, a simple cyclic voltammetric scanning between –0.5 and 1.0 V took place until a cyclic oltammogram characteristic of a clean Pt electrode was obtained.

Optimization of separation and detection conditions

Sample was injected by electrokinetic mode for 10 s at 10 kV. Separation voltage was set at 15 kV. A 10.0 mM phosphate buffer was used as running buffer. Prior to starting a series of analyses, the capillary was washed with 0.1 M sodium hydroxide for 5 min, followed by double-distilled water for 5 min, and equilibrated with the running buffer for 5 min so as to maintain an active and reproducible inner surface. The voltage of photomultiplier tube for collecting the ECL signal was set at 800 V in the process of detection. ECL in the reservoir, a 5 mM $Ru(bpy)_3^{2+}$, and 50 mM phosphate buffer were replaced once for 4 h. Every run was repeated at least three times under the same conditions.

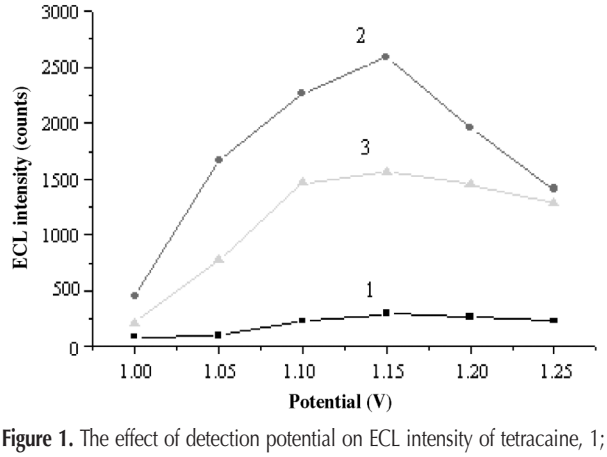

Figure 1. The effect of detection potential on ECL intensity of tetracaine, 1; proline, 2; and enoxacin, 3. Conditions: 5 mM $Ru(bpy)_3^{2+}$; 50 mM phosphate buffer (pH = 8.00); electrokinetic injection, 10 s at 10 kV; separation phosphate buffer (pH = 9.60), 10 mM; separation voltage, 15 kV. Data point is the average value of three measurements and contains less than 3% of relative standard deviation.

Results and Discussion

Optimization of detection conditions

Effect of detection potential

The detection potential was carefully evaluated to achieve a maximum ECL signal. The influence of applied potential on the analyte ECL signals was tested by changing the potential from +1.00 to +1.25 V. As shown in Figure 1, tetracaine, proline, and enoxacin displayed similar profiles in the studied potential range. Because the oxidation of $Ru(bpy)_3^{2+}$ needs to be at least +1.00 V, when the applied potential was less than +1.00 V, the ECL emission was hardly observed. With the increasing applied potential from +1.00, the ECL intensities for tetracaine, proline, and enoxacin increased, reached the maximum values at +1.15 V, and then decreased slightly. Therefore, +1.15 V was adopted as the optimum detection potential in following experiments.

Effect of TBR concentration

The electrogenerated $Ru(bpy)_3^{3+}$ was a stoichiometrically limiting reagent for the ECL reaction [$Ru(bpy)_3^{3+}$–amine, 2:1, v/v] (42). Increasing the concentration of $Ru(bpy)_3^{2+}$ resulted in the increase of ECL intensity. However, the background noise increased with increasing $Ru(bpy)_3^{2+}$ concentration. Moreover, $Ru(bpy)_3^{2+}$ was expensive from a practical point of view. The concentration of $Ru(bpy)_3^{2+}$ was fixed at 5 mM for the determination of atenolol and metoprolol with a high signal-to-noise ratio (43). Investigation of the effect of the concentration of $Ru(bpy)_3^{2+}$ on the three analytes ECL intensity from 1 to 5 mM indicated that the three analytes had similar profile of ECL intensity versus concentration of $Ru(bpy)_3^{2+}$. Although higher concentration of $Ru(bpy)_3^{2+}$ showed a larger response, it also yielded more noise and consumed larger amount of the expensive reagent. This might result from the more rapid CE reaction kinetics due to the increased ratio of reagent to analyte (44). In order to maintain high sensitivity, low baseline noise and a moderate reagent consumption, a 5 mmol/L $Ru(bpy)_3^{2+}$ contained in the 50 mmol/L phosphate salt solution at pH 8.00 was used for the ECL in the detection cell. After an operation for 3–4 h, the $Ru(bpy)_3^{2+}$ solution needed to be replenished in order to maintain good reproducibility.

Effect of buffer pH and concentration

The pH value of ECL solution is an important factor affecting the ECL intensity (40). As previously reported, the ECL intensity of the $Ru(bpy)_3^{2+}$/amine system strongly depended on the pH value at which the reaction was conducted. The optimum signal-to-noise ratio could be obtained when the buffer pH was slightly basic (45). The efficiency was markedly affected by both reaction pH and the analyte molecular structure. The effect of pH on the ECL intensity was investigated in the pH range of 4.5–11.5 in 0.50 pH units. Figure 2 shows the effect of pH value on the ECL intensity of the analytes. From the result, we can see the ECL intensity of tetracaine changed slightly in the pH range, but at very low pH values the analytes radical cation is difficult to deprotonate to form high reducing free radical intermediate. The ECL intensity of proline and enoxacin apparently increased with the pH value until an ECL intensity peak appeared at the pH value of 8.00. When the pH value was higher than 8, the ECL intensity was slightly decreased. It is possible that the reaction of $Ru(bpy)_3^{3+}$ and OH consumed the reactant with increasing pH value, and thus resulted in a decrease of the ECL intensity (46). As the results show earlier, the solution at pH 8.00 was chosen with a compromise.

Investigation of the concentration of the buffer in the detection cell was also performed. The concentration changed from 20 to 80 mM (pH = 8.00). When the concentration of the running buffer was 50 mM, the highest ECL intensity of tetracaine, proline, and enoxacin was obtained. When the concentration of detection buffer was too low (< 50 mM), the electronic transfer in ECL reaction was limited. As the concentration of background electrolyte is too high, the quantity of $Ru(bpy)_3^{2+}$ ions in the vicinity of the working electrode will be reduced because other ions may replace $Ru(bpy)_3^{2+}$ near the electrode. The two factors reach a compromise at a concentration of 50 mM.

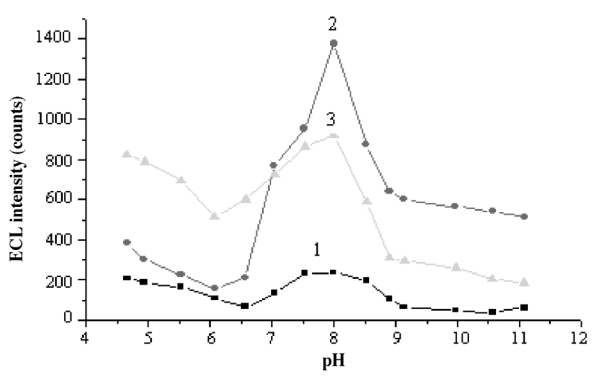

Figure 2. The effect of pH of the phosphate buffer in ECL cell on ECL intensity of tetracaine, 1; proline, 2; and enoxacin, 3. Conditions: 5 Mm $Ru(bpy)_3^{2+}$, 50 mM phosphate buffer; electrokinetic injection, 10 s at 10 kV; separation phophate buffer (pH = 9.60), 10 mM; separation voltage, 15 kV. Data point is the average value of three measurements and contains less than 3% of relative standard deviation.

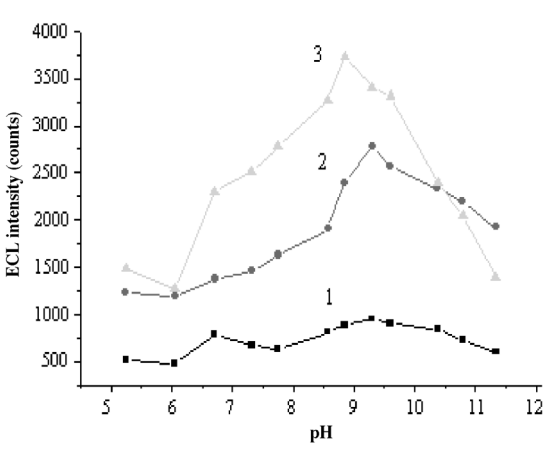

Figure 3. The effect of pH of the phosphate buffer in capillary on ECL intensity of tetracaine, 1; proline, 2; and enoxacin, 3. Conditions: 5 mM $Ru(bpy)_3^{2+}$; 50 mM phosphate buffer(pH = 8.00); electrokinetic injection, 10 s at 10 kV; separation phosphate buffer, 10 mM; separation voltage, 15 kV. Data point is the average value of three measurements and contains less than 3% of relative standard deviation.

Optimization of separation conditions

Effect of buffer pH for separation

The pH values of the running buffer strongly influence the ECL intensity due to directly affect electroosmosis flow (41). Therefore, it is vital to investigate its influence on CE in order to obtain optimum separations. The variety of the ECL intensity in the pH range of 5–11.5 was studied as shown in Figure 3. When pH > 8.57 for the buffer the peaks of tetracaine, proline, and enoxacin were completely separated. The intensity of ECL is not highest at pH 9.6, but the separation is better than any other. Considering some major parameters, such as ECL intensity and effective separation as well as migration time, the pH value of running buffer was optimized at 9.60.

Effect of separation buffer concentration

With a fixed pH value at 9.60, the buffer concentration was changed from 5 to 40 mM. The effect of running buffer concentration on ECL intensities and the resolution of the three drugs were investigated, and the results are shown in Figure 4. When the concentration was below 10mM, lower ECL intensities and resolution were obtained as a result of the lower ionic strength. The highest ECL signal was observed when the buffer concentration was 20 mM. We also found that with the increase of the buffer concentration, the migration time became longer because of the electroosmotic flow decreased. At the same time the ECL baseline became unstable and the peaks were to be broadened. This was due to the effect of the increase of the ionic strength, which resulted in the increase of Joule heating. Considering all influence factors, the concentration of running buffer set at 20 mM for further experiment.

Effect of separation voltage

A study of the influence of separation voltage on the emission intensities was carried out from 5 to 20 KV. ECL intensity increased with separation voltage increased up to 15 kV, then it reached a plateau. In CE–ECL system, as separation voltage increased, the electroosmosis flow should be increased, thus more analyte in the effluent arrives in the diffusion layer of working electrode within a given time and higher ECL signal and shorter migration time could be obtained. On the other hand, higher voltage would cause a higher current and lead to more Joule heating, which affected the separation of the tested analytes (11). Futhermore, the analytes may not have enough time or space to react with oxidized $Ru(bpy)_3^{3+}$ because of the strong flow rate of effluent, thereby making the efficiency of light producing reaction invariable. Considering all these factors, 15 kV was chosen as a separation voltage in our experiment to ensure high ECL intensity and good repeatability.

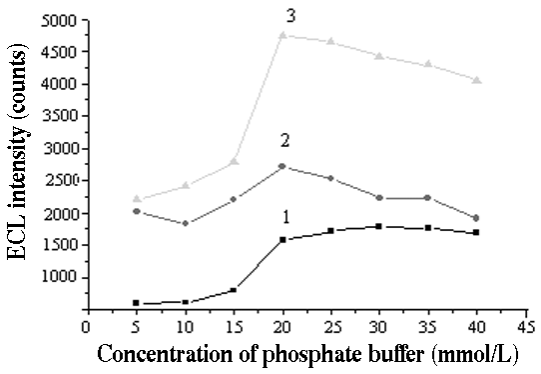

Figure 4. The effect of concentration of the phosphate buffer in capillary on ECL intensity of tetracaine, 1; proline, 2; and enoxacin, 3. Conditions: 5 mM $Ru(bpy)_3^{2+}$; 50 mM phosphate buffer (pH = 8.00); electrokinetic injection, 10 s at 10 kV; separation phosphate buffer (pH = 9.60); separation voltage, 15 kV. Data point is the average value of three measurements and contains less than 3% of relative standard deviation.

Mechanism of ECL

CL reaction occurs in the diffusion layer near the electrode when the active $Ru(bpy)_3^{3+}$ species were electrochemically generated from the inactive $Ru(bpy)_3^{2+}$ at the electrode surface (24). It has been shown that a wide range of primary, secondary, and tertiary amines as well as amino acids will react with $Ru(bpy)_3^{3+}$ to yield CL (24–31,33–42,45–47). Tetracaine, enoxacin, and proline were reducing agents, and their structures are as shown in Figure 5.

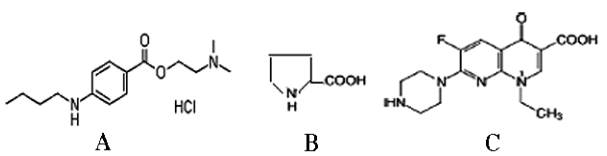

Figure 5. Structure of tetracaine (A), proline (B), and enoxacin (C).

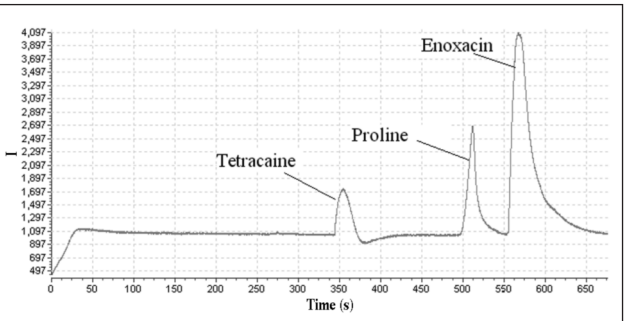

Figure 6. Electropherograms of tetracaine, proline, and enoxacin in spiked urine sample. Conditions: 5 mM $Ru(bpy)_3^{2+}$; 50 mM phosphate buffer (pH = 8.00) in the detection reservoir; electrokinetic injection, 10 s at 10 kV; separation phosphate buffer 20 mM (pH = 9.60); separation voltage, 15 kV.

Table I. Determination Results and Recovery Rates in Urine Samples

Compound	Added (μg/mL)	Found (μg/mL)	Recovery (%)	% RSD (*n* = 5)
Tetracaine	0.50	0.47	94.0	3.1
	4.00	3.74	93.5	3.3
	18.0	17.1	95.0	3.6
Proline	0.50	0.48	96.0	2.9
	4.00	3.90	97.5	3.0
	18.0	17.7	98.3	3.5
Enoxacin	0.50	0.48	96.0	3.4
	4.00	3.93	98.3	3.2
	18.0	17.5	97.2	3.1

The second amino group in tetracaine molecule, amino group (similar to second amino group) in proline molecule, and piperazine ring in enoxacin molecule are oxidized by the generated $Ru(bpy)_3^{3+}$, which transformed to a luminant, $Ru(bpy)_3^{2+*}$. The mechanism of electrochemical CL for this system is likely to one for $Ru(bpy)_3^{2+}$/TprA system (48), which can be expressed as follows:

$Ru(bpy)_3^{2+} - e^- \rightarrow Ru(bpy)_3^{3+}$ (Anode)
$Ru(bpy)_3^{3+} + \text{analyte} \rightarrow Ru(bpy)_3^{2+} + \text{analyte}^{\bullet+}$
$\text{analyte}^{\bullet+} \rightarrow \text{analyte}^{\bullet} + H^+$
$Ru(bpy)_3^{3+} + R_3N^{\bullet} \rightarrow Ru(bpy)_3^{2+*} + H^+ + \text{by-products}$
$Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + h\nu$

Repeatability, linearity, and detection limit

Under the optimized conditions: ECL detection at 1.15 V, 20 mM phosphate buffer at pH 9.60, 5 mM $Ru(bpy)_3^{2+}$, and 50 mM phosphate buffer at pH 8.00 in the detection reservoir, a standard mixture solution containing 6 µg/mL tetracaine, proline, and enoxacin was injected consecutively eleven times to determine the repeatability of ECL intensity based on their peak height and migration time. Relative standard deviations of the ECL intensity and the migration time were 3.8 and 1.3% for tetracaine, 3.4 and 1.0% for proline, as well as 3.6 and 1.2% for enoxacin, respectively. The high repeatability indicates that this approach is accurate for detection of tetracaine, proline, and enoxacin.

To investigate the detection linearity by CE–ECL, a series of standard mixture solutions containing three species were tested. The calibration curves of peak height response versus analyte concentration were linear in the range of 0.4–100 µg/mL for tetracaine, 0.2–80 µg/mL for proline, and 0.1–100 µg/mL for enoxacin with correlation coefficients of 0.996, 0.998, and 0.997, respectively.

Detection limits (signal-to-noise ratio = 3) of 0.08 µg/mL for tetracaine, 0.06 µg/ mL for proline, and 0.02 µg/mL for enoxacin were obtained. The detection limits of the proposed method were lower than those of HPLC (0.5 µg/mL) (8), micellar LC (0.025 µg/mL) (9), CE (11), and ECL (0.26 µg/mL) (12) for enoxacin; spectrophotometry (18.0 µg/mL) (13) and CL (0.1 µg/mL) (17) for tetracaine; and CE–ECL (0.43µg/mL) (23) and CE–ECL with $Ru(bpy)_3^{2+}$ (0.88 µg/mL, 0.22 µg/mL) (38–40) for proline. The obtained detection limits permit the detection of the three analytes in urine samples for understanding the patients' medical process.

Application to human urine

All urine samples were treated as shown in the Standard solution and sample preparation section and analyzed with the CE–ECL system. Electropherograms of spiked urine sample are shown in Figure 6. A baseline separation for tetracaine, proline, and enoxacin was achieved within 10 min.

The proline concentration of 13.7–26.0 µg/mL in human urine was reported (40). ENX has been found to be 1.125– 2.787 µg/mL in urine a few hours after oral administration, and a single oral dose of 200 mg was given (49). The tetracaine concentration in human plasma was found to be 0.7 µg/mL (50), and no data in urine was reported. According to these data, the recoveries were investigated. The calibration curves were generated with the standard solutions of 0.4, 1.0, 3.0, 6.0, 10.0, 15.0, 20.0, and 25.0 µg/mL of tetracaine, enoxacin, and proline. The content of proline in human urine is in the range of 798.2–893.7 µg/mL. The recovery and relative standard deviation (RSD) are given in Table I. The recoveries were 93.5–95.0% for tetracaine, 96.0–98.3% for proline, and 96.0–98.3% for enoxacin, and the relative standard deviation (n = 5) was in the range of 3.1–3.6% for tetracaine, 2.9–3.5% for proline, and 3.1–3.4% for enoxacin.

Conclusion

A simple, fast, and sensitive method has been developed for simultaneous determination of tetracaine, proline, and enoxacin in urine. Under the optimized conditions, the approach of CE–ECL with $Ru(bpy)_3^{2+}$ showed good performance in terms of selectivity, sensitivity, repeatability, analysis time, and linearity. The validated method could be a good tool for the assay of tetracaine, proline, and enoxacin in urine.

Acknowledgements

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