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Capillary electrophoresis with electrochemiluminescence detection for simultaneous determination of proline and fleroxacin in human urine

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The content of free proline (Pro) in body fluids is a biological parameter for patients with renal insufficiency and chronic uraemia. Fleroxacin (FLX) must be used cautiously because of adverse reactions. Its dosage must be adjusted according to the degree of renal insufficiency. In a clinical setting the simultaneous determination of Pro and FLX in body fluids is necessary for the rational utilization of FLX. A capillary electrophoresis (CE) method based on electrochemiluminescence (ECL) detection with $Ru(bpy)_3^{2+}$ was developed for the simultaneous determination of Pro and FLX in human urine. Parameters related to separation and detection were investigated and optimized. The most favourable resolution and high sensitivity were obtained using a 15 mM phosphate buffer at pH 9.6 with the detection potential at 1.15 V. Under optimized conditions, the standard curves were linear in the range of 0.1–80 μg/mL for Pro and 0.1–100 μg/mL for FLX. Detection limits(3σ) of 0.3 ng/mL for FLX and 0.02 μg/mL for Pro were obtained. Relative standard derivations (RSDs) of the ECL intensity and the migration time were 3.2% and 0.9% for 4 μg/mL Pro and 3.7% and 1.2% for 4 μg/mL FLX, respectively. The recoveries were in the range of 94.8–99.6% for Pro with RSDs of 2.8%–3.6% and 94.7–97.8% for FLX with RSDs of 2.9%–3.7%. The proposed method was successfully applied to determine the amounts of Pro and FLX in human urine.

Keywords: capillary electrophoresis; electrochemiluminescence; proline; fleroxacin; human urine

Introduction

Proline (Pro) presents in biological fluids in free, peptide and protein forms. Its contents vary in association with various diseases such as bone diseases, tumours and chronic uraemia.[1] The content of free proline (Pro) in body fluids is a biological parameter for patients with renal insufficiency and chronic uraemia. Its content in body fluids for patients is higher than that for healthy people.[2] Fleroxacin (FLX) is a new fluoroquinolone antibiotic that exhibits strong bactericidal activity against a wide range of Gram-negative and Gram-positive bacteria.[3] However, fleroxacin was associated with a lot of adverse drug reactions and must be used cautiously for patients with renal insufficiency.[4] The dosage must be adjusted according to the degree of renal insufficiency. If the dosage is too big it will cause haematuria. It may also inhibit DNA replication, recombination, repair and transcription. Thus it is important to control FLX dosage and monitor it in body fluids; otherwise, before and after oral administration of FLX, the monitoring of proline content in body fluids is necessary for patients with renal insufficiency or chronic uraemia. Therefore, developing a simple and efficient method for simultaneous determination of Pro and FLX is an important analytical task.

Several methods have been described for the determination of Pro by liquid chromatography,[1] spectrophotometry[5] and chemiluminescence (CL).[6] Several analytical procedures have been reported for the determination of FLX, including spectrofluorometry,[7,8] gas chromatography (GC),[9] high performance liquid chromatography(HPLC),[10–13] spectrophotometry,[14,15] an electroanalysis method,[16,17] and a CL method.[18,19] However, these methods require more sophisticated instrumentation, or lower sensitivity, or are more time consuming. Thus, it is very important to develop a simple and rapid method for simultaneous detection of Pro and FLX.

Increasingly, capillary electrophoresis (CE) has become an efficient separation technique. Separations with several hundred thousand theoretical plates have been achieved even with simple capillary zone electrophoresis in its early days.[20] The high-efficiency, powerful resolution, fast separation, low instrumental cost and low consumption of samples and reagents are the main advantages of CE over HPLC.[21] Capillary electrophoresis has been used for the separation and determination of FLX with UV detection.[22,23] High-performance CE was used to determine FLX in human urine.[24] The development of electrochemiluminescence (ECL) detection for CE has been critically reviewed.[25] Electrochemiluminescence detection, in comparison with other modes, offers lower background noise, higher detection sensitivity and requires simple and inexpensive instrumentation. Capillary electrophoresis coupled with ris(2,2′-bipydidyl)Ruthenium(II) ($Ru(bpy)_3^{2+}$) ECL detection has been applied in the determination of human urinary proline and hydroxyproline with the detection limit of 4 μmol/L.[26] However, until now, there has been no

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report of the determination of FLX by the CE-ECL method and simultaneous determination of Pro and FLX.

The aim of this study was to develop an efficient CE method for simultaneous determination of Pro and FLX by using 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 Pro and FLX in human urine with satisfactory results.

Experimental

Reagents and chemicals

Pro was obtained from Sigma(USA). Tris(2,2′-bipydidyl) ruthenium(II) chloride hexahydratewas (TBR) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The FLX was obtained from the Institute of Medical Biotechnology (Beijing, China). Standard stock solutions of Pro and FLX were prepared with double-distilled water, and stored at 4 °C in a refrigerator. Working standard solutions were prepared by precise dilution of standard stock solutions with double-distilled water. The phosphate buffer used in the detection cell and the electrophoresis separation buffer was prepared by mixing equimolar (5–80 mM) amount of disodium hydrogenphosphate and sodium dihydrogenphosphate. The appropriate pH value (4.5–11.5) of the buffer was adjusted with orthophosphoric acid or sodium hydroxide. All chemicals, including phosphate, sodium hydroxide were of analytical grade. The double-distilled water was prepared using Mili-Q ultra-high purity water system (XGJ-30 water purified system, Yongcheng Company in Beijing, China). Prior to CE analysis, the drug solution and buffer were filtered through a 0.22 μm membrane before use.

CE–ECL system

All experiments were performed using a computer-controlled CE–ECL system (Xi'an Remax Electronics Co. Ltd, Xi'an, China), which included a high-voltage power supply for electrophoretic separation and electrokinetic injection, a potential control system, a chemiluminescence 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. End-column detection was employed using a wall-jet configuration. Separation voltage was set at 15 kV. In the reservoir, a solution of 5 mM $Ru(bpy)_3^{2+}$ and 50 mM phosphate buffer were replaced once every 4 hours. Separations were performed in 50 cm 25 μm i.d and 360 μm o.d. long fused silica capillaries (Yongnian Optical Fabric Factory Hebei, China). The capillary was filled with 0.1 M sodium hydroxide and allowed to equilibrate over night. Prior to starting a series of analysis, the capillary was washed with 0.1 M sodium hydroxide for 5 minutes, followed by double-distilled water for 5 minutes, and equilibrated with the running buffer for 5 minutes so as to maintain an active and reproducible inner surface. The voltage of the photomultiplier tube was set at 800 V for collecting the ECL signal. The sample solution was injected by electromotion for 10 s at 10 kV. Each sample was performed in triplicate.

Sample analysis

Five healthy volunteers were treated simultaneously with an oral administration of 200 mg FLX capsule. The subjects included a 60-year-old male weighing 65 kg, two 23-year-old males weighing 60 kg, and two 22-year-old females with 50 kg body weight. The urine samples were collected at 1, 4, 6, 8, 12, 24 h after oral administration. A 1 mL urine sample was diluted with 10 mL of double-distilled water and 0.5 mL of the diluted urine sample was deproteinized by adding 0.5 mL 10% trichloroacetic acid (CCl_3COOH) in a 1.5 mL centrifuge tube, which was then centrifuged for 15 minutes at 4000 rpm. The centrifugate was used for CE-ECL analysis. The optimized conditions were: ECL detection at 1.15 V, 15 mM phosphate buffer at pH 9.6, 5 mM $Ru(bpy)_3^{2+}$ and 60 mM phosphate buffer at pH 7.5 in the detection reservoir. The calibration curves were generated with the standard solutions of 40, 50, 60, 70, 80 and 90 μg/mL of Pro and 2, 4, 6, 8 and 10 μg/mL of FLX. Under the same conditions, the Pro and FLX content of the urine sample collected at different times was determined. After each determination, a 50 μg/mLstandard solution of Pro and FLX was added in the urine sample. The spiked urine samples were analysed and the recoveries were calculated.

Results and Discussion

Optimization of detection conditions

Effect of detection potential on detection

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. Figure 1 illustrates that the highest ECL intensity was at 1.15 V, as shown in the hydrodynamic voltammograms, hence, the most sensitive detection potential for Pro and FLX was 1.15 V.

Effect of the phosphate buffer pH on detection

The efficiency of ECL is affected markedly by both reaction pH and the analyte molecular structure. The deprotonation of analyte in the ECL reaction is an important factor affecting the ECL intensity. The effect of the pH on ECL intensities of the two analytes was tested with 50 mM phosphate buffer in the pH range from 4.5 to 11.5.

Figure 2 indicates that the ECL intensity of Pro increased with increasing pH value until an ECL intensity peak appeared at the

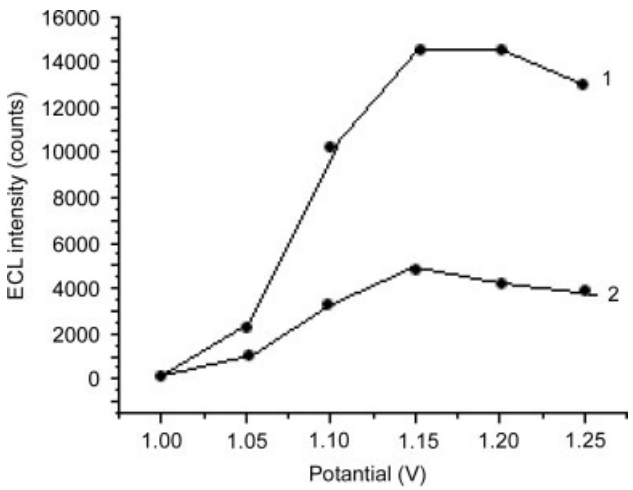

Figure 1. Effect of detection potential on ECL intensity of 80 μg/mL Pro(1) and 80 μg/mL FLX(2); 5 mM $Ru(bpy)_3^{2+}$; 50 mM phosphate buffer; electrokinetic injection, 10s at 10 kV. Data point is the average value of three measurements with <3% of RSD.

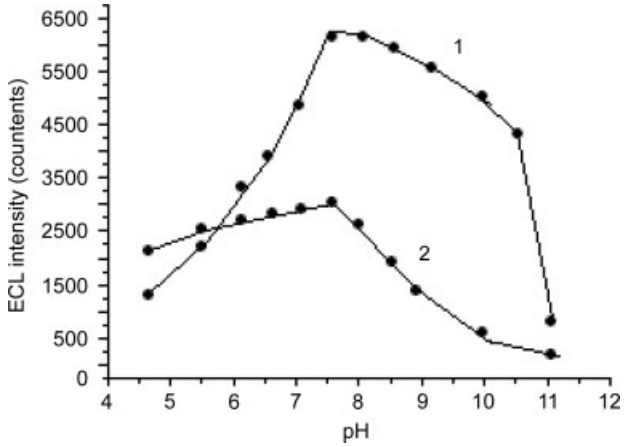

Figure 2. Effect of pH of the phosphate buffer in ECL cell on ECL intensity of 80 μg/mL Pro (1) and 80 μg/mL FLX. (2); 5 mM $Ru(bpy)_3^{2+}$; 50 mM phosphate buffer; electrokinetic injection, 10 s at 10 kV; Data point is the average value of three measurements with <3% of RSD.

pH value of 7.5, then decreased slightly. The ECL intensity of FLX increased with the increase of pH value until an ECL intensity peak appeared at the pH value of 8.0, then decreased slightly. At very low pH values the analytes radical cation is difficult to deprotonate to form a high reducing free radical intermediate. At high pHs the lower ECL intensity resulted from the reduced availability of $Ru(bpy)_3^{3+}$ due to the competitive reaction with hydroxide ion which presents considerable concentration levers at high pHs.[27] The results demonstrated that the ECL intensity depends on the structures of analytes and the detection buffer pH. Therefore, the buffer pH value was set at 7.5 in this study.

Effect of the phosphate buffer concentration on detection

Another investigation of the pH 7.5 buffer concentration from 20 to 80 mM in the detection cell was also performed. The highest ECL intensity of Pro and FLX was obtained when the concentration of the buffer was 60 mM. If the ionic strength of background electrolyte was too low, transfer of electrons produced in the electrochemical steps would be slowed, resulting in the decreased ECL efficiency. When the concentration of the buffer was above 60 mM,, 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, moreover, the CL baseline became unstable. The concentration of the buffer was set at 60 mM.

Effect of TBR concentration on detection

The concentration of $Ru(bpy)_3^{2+}$ added in the detection cell is one of the most important detection parameter. A higher sensitivity was obtained with increasing the concentration of $Ru(bpy)_3^{2+}$, but at the same time the background noise increased and larger amount of the expensive reagent was consumed. To obtain a higher S/N value, ECL efficiency and a moderate reagent consumption, a 5 mM $Ru(bpy)_3^{2+}$ was used in our experiment. After operating for 3–4 hours, the $Ru(bpy)_3^{2+}$ solution was replenished in order to maintain good reproducibility.

Optimization of separation conditions

Effect of separation buffer pH

The separation buffer pH influences not only the charge of the analytes but also the electro-osmotic flow inside the capillary, which, in turn, results in different migration times for analytes. Therefore, it is vital to investigate its influence on CE in order to obtain optimum separations. The resolution (*Rs*) between Pro and FLX is calculated with the following equation: $Rs = 2(t_2 - t_1)/(W_{b1} + W_{b2})$, where t_1 and t_2 are migration times of Pro and FLX, respectively, and W_{b1} and W_{b2} are the peak widths of Pro and FLX measured at the baseline. When the buffer pH from 5 to 11.5 was used, the peaks of Pro and FLX were completely separation (Rs >2). The ECL intensity of Pro and FLX was observed when the separation buffer pH was in the range of 5.0–11.5 (Fig. 3).

The ECL intensity was increased dramatically with the buffer pH value increased up to 9.6, and when the buffer pH exceeded 9.6, the ECL response would be decreased. Considering some major parameters such as ECL intensity and maximum separation as well as migration time, the pH 9.6 separation buffer was used in the analysis.

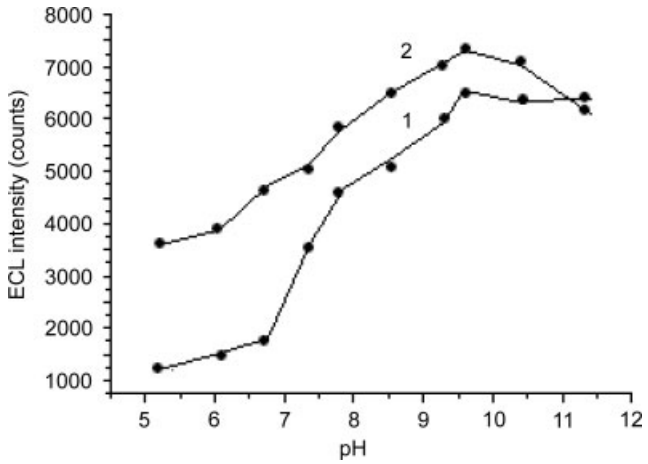

Figure 3. The effect of pH of the separation buffer in capillary on ECL intensity of 80 μg/mL Pro (1) and 80 μg/mL FLX (2); 5 mM $Ru(bpy)_3^{2+}$; separation phosphate buffer, 10 mM; separation voltage, 15 kV. Data point is the average value of three measurements with <3% of RSD.

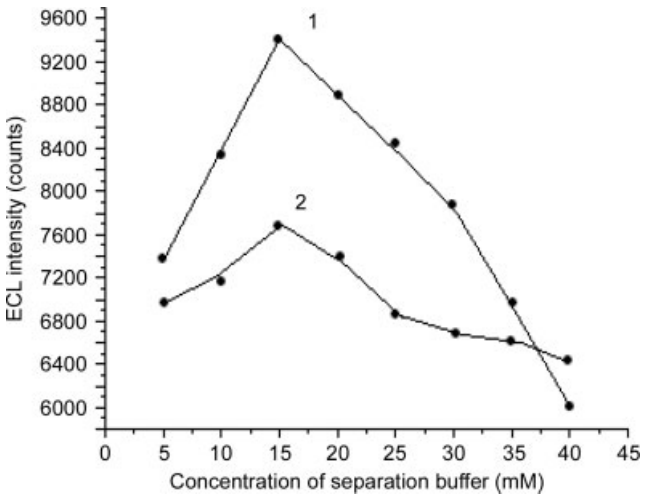

Figure 4. Effect of concentration of the separation buffer in capillary on ECL intensity of 80 μg/mL Pro(1) and 80 μg/mL FLX (2); 5 mM $Ru(bpy)_3^{2+}$; separation phosphate buffer (pH = 9.6); separation voltage, 15 kV. Data point is the average value of three measurements with <3% of RSD.

Effect of separation buffer concentration

The effect of the concentration of separation buffer (5–40 mM) on ECL intensity and the separation of the Pro and FLX was tested using a pH of 9.6 in the buffer solution, as shown in Fig. 4.

Over the concentration range of 5–40 mM, the two species were sufficiently separated. However, the migration time of each individual species increased with the increase in the buffer concentration. If the buffer concentration was lower than 10 mM, insufficient buffer capacity would be given. The concentration at 15 mM was found to be optimal for the separation of Pro and FLX in the CE–ECL system.

Effect of separation voltage

In the CE/ECL system, the influence of separation voltage on the emission intensity was investigated from 5 kV to 25 kV. Electrochemiluminescence intensity increased with separation voltage increased up to 15 kV. The electro-osmosis flow should increase with increasing separation voltage, thus more analyte in the effluent appears in the diffusion layer of the working electrode within a given time and a higher ECL signal can be obtained. On the other hand, the strong flow of effluent from the capillary may reduce the concentration of $Ru(bpy)_3^{3+}$ at the electrode surface, thereby reducing the efficiency of the light-producing reaction. Therefore, when separation voltage ranged from 15 kV to 25 kV, the ECL signal did not increase, and reached a plateau. A 15 kV was chosen as a separation voltage in our experiment to ensure high ECL intensity and good reproducibility.

Mechanism of ECL

The ECL 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. A double bond C═O on the ring in FLX molecular and the ring in the Pro molecule are easily oxidized by the generated $Ru(bpy)_3^{3+}$. The ECL mechanism can be expressed as follows:

$$Ru(bpy)_3^{2+} \longrightarrow Ru(bpy)_3^{3+} + e^-(\text{Anode})$$
$$Ru(bpy)_3^{3+} + \text{Pro} \longrightarrow Ru(bpy)_3^{+} + \text{Oxidised Pro}$$
$$Ru(bpy)_3^{3+} + \text{FLX} \longrightarrow Ru(bpy)_3^{+} + \text{Oxidised FLX}$$
$$Ru(bpy)_3^{+} + Ru(bpy)_3^{3+} \longrightarrow Ru(bpy)_3^{2+*} + Ru(bpy)_3^{2+}$$
$$Ru(bpy)_3^{2+*} \longrightarrow Ru(bpy)_3^{2+} + e^- (\text{Anode}) + h\nu\ (620\ \text{nm})$$

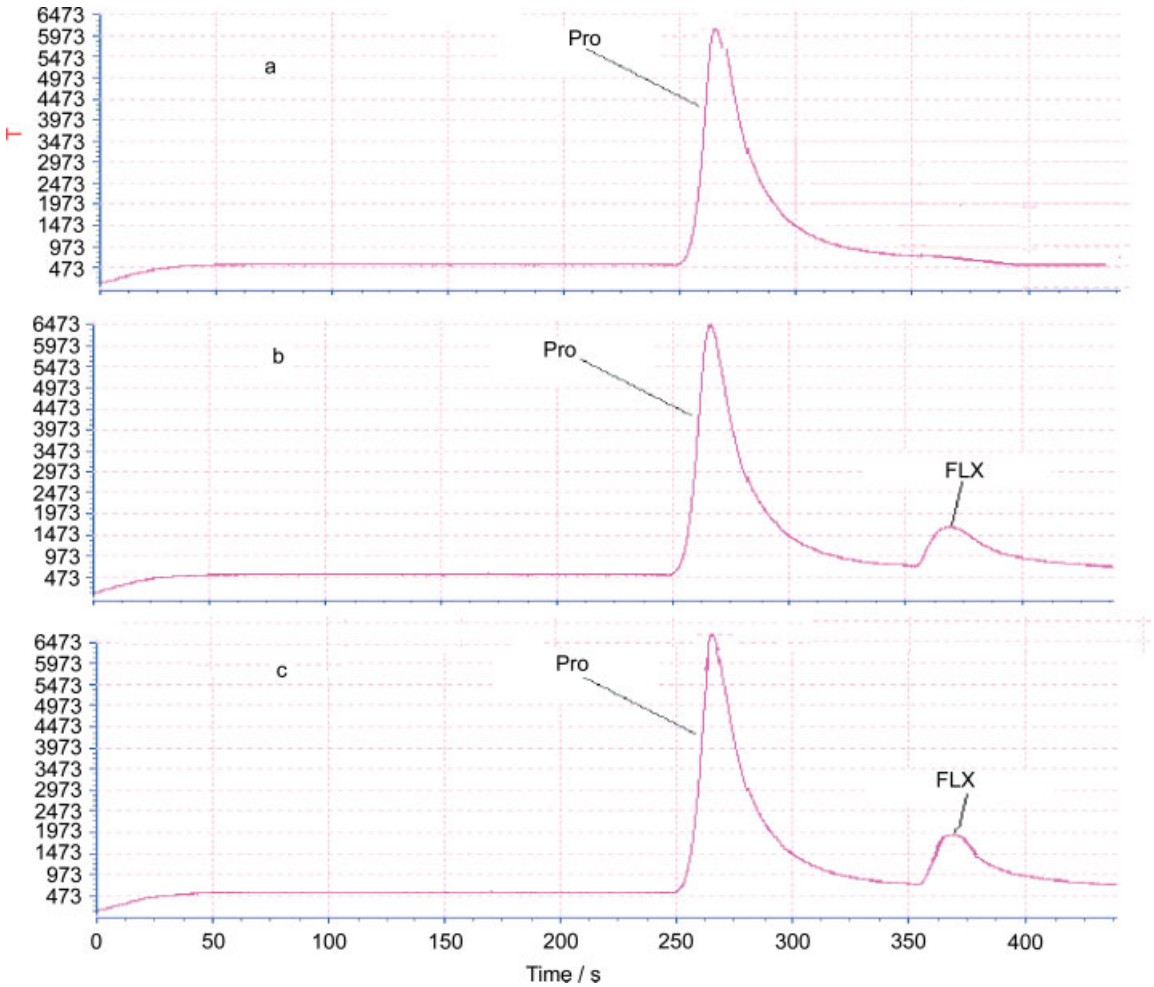

Figure 5. Electropherograms of Pro and FLX in (a) urine sample before oral admission of FLX, (b) urine sample at 12 hours after oral admission of FLX and (c) mixed standard solution; 5 mM $Ru(bpy)_3^{2+}$; 60 mM phosphate buffer(pH = 7.5) in the detection reservoir; electrokinetic injection, 10 s at 10 kV; separation phosphate buffer 15 mM (pH = 9.6); separation voltage, 15 kV.

Table 1. Determination of Pro and FLX in human urine samples

Analyte	Time (h)	Content* (μg/mL)	Added (μg/mL)	Found (μg/mL)	Average recovery (%)	RSD n = 5 (%)
Pro	0	893.2	50	49.7	99.4	3.6
	1	894.0	50	49.8	99.6	3.5
	4	893.7	50	49.4	98.8	3.4
	6	891.0	50	49.8	99.6	2.8
	8	854.1	50	47.8	95.7	3.0
	12	798.2	50	47.4	94.8	3.6
	24	823.4	50	48.7	97.4	3.1
FLX	1	**	50	49.7	99.4	3.4
	4	41.6	50	49.8	99.6	3.2
	6	88.3	50	49.7	99.4	2.9
	8	68.2	50	49.5	99.0	3.2
	12	31.7	50	49.6	99.2	3.5
	24	18.3	50	49.8	99.6	3.7

* Average content for five healthy volunteers, ** not detected

Reproducibility, linearity, detection limit

Under optimized conditions, a standard mixture solution containing 4 μg/mL Pro and FLX was injected consecutively 11 times to determine the reproducibility of ECL intensity based on peak height and migration time for Pro and FLX. Relative standard derivations of the ECL intensity and the migration time were 3.6 and 1.0% for Pro and 3.3 and 1.3% for FLX, respectively. The high reproducibility indicates that this approach is accurate for detection of Pro and FLX.

To investigate the detection linearity of Pro and FLX, a series of standard mixture solutions containing the two species were tested. The standard curves were linear in the range of 0.1–80 μg/mL for Pro and 0.1–100 μg/mL for FLX. The calibration equations and regression coefficients were $y = 111.5x + 171.3$ and $R = 0.998$ for Pro, $y = 81.4x - 160.1$ and $R = 0.997$ for FLX in terms of peak height response as a function of analyte concentration.

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a height three times the level of the baseline noise. The LOD (3σ) of 0.3 ng/mL for FLX and 0.02 μg/mL for Pro were obtained. The LOD of the proposed method for FLX was lower than those of fluorescence spectrometry (90, 24 ng/mL),[7,8] HPLC (1.0–58 ng/mL),[10–13] spectrophotometry (58 ng/mL),[14] and voltammetric method(74 ng/mL).[17] CL method (9 ng/mL),[19] and CE (2.3 μg/mL).[24] The LOD for Pro was lower than that of CE-ECL method (0.64 μg/mL for Pro).[26] This indicated that the proposed CE-ECL procedure has good linearity, higher sensitivity and precision.

Application to human urine

The proposed CE-ECL method was applied to determine Pro and FLX in the human urine under the optimized conditions. The five healthy volunteers were treated simultaneously with an oral administration of 200 mg FLX capsule. The urine samples were collected at different periods of time after oral administration of FLX for determination of FLX and Pro. The urine collected before the dosing was employed as a blank for determination of FLX. All urine samples were treated as shown in the sample analysis section and examined with CE-ECL system. Electropherograms of standard and urine sample are shown in Fig. 5.

The contents of Pro and FLX in urine samples collected at different time distances were determined. At the same time, the recoveries of Pro and FLX from each urine sample spiked with 50 μg/mL analyte were determined. The results for the mean contents and recoveries of Pro and FLX at different time distances for five healthy volunteers are summarized in Table 1, along with the relative standard deviation (RSD). The recoveries were in the range of 94.8–99.6% for Pro with the RSDs of 2.8–3.6% and 94.7–97.8% for FLX with the RSDs of 2.9–3.7%. The content of Pro in the urine samples for the elder was lightly higher than that for the youngsters. The content of Pro in the urines before and after oral administration for the five healthy volunteers seemed kept constant, it is was accordant with the report in literature.[28] The highest content of FLX was observed in the urine selected 6 hours after oral administration.

Conclusion

A new method has been developed for simultaneous determination of Pro and FLX in human urine. The CE-ECL approach with $Ru(bpy)_3^{2+}$ performed well in terms of selectivity, sensitivity, repeatability, short analysis time and linearity. The validated method can be used for routine determination of Pro and FLX in human urine.

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