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Simultaneous Determination of Proline and Pipemidic Acid in Human Urine by Capillary Electrophoresis With Electrochemiluminescence Detection

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Pipemidic acid is extensively used in the treatment of Gram-negative urinary tract infections, and the contents of proline in human urine vary in association with chronic uremia. The simultaneous determination of pipemidic acid and proline in human urine is of significance for quality control of the dosage and clinical study. The coupling of $Ru(bpy)_3^{2+}$-based electrochemiluminescence detection with capillary electrophoresis was developed for the simultaneous determination of proline and pipemidic acid in human urine. Parameters related to the separation and detection were investigated and optimized. The standard curves were linear between 0.1 and 90 $\mu g\,mL^{-1}$ for proline and between 0.4 and 100 $\mu g\,mL^{-1}$ for pipemidic acid. Underoptimized conditions, the detection limits (3σ) were 0.02 $\mu g\,mL^{-1}$ for proline and 0.06 $\mu g\,mL^{-1}$ for pipemidic acid. Relative standard derivations for the electrochemiluminescence intensity and the migration time were 3.2 and 0.9% for proline and 3.7 and 1.2% for pipemidic acid, respectively. The developed method was successfully applied to determine proline and pipemidic acid in human urine. The result showed that the content and decreasing rates of proline in urine for male were higher than that for female, and the content and decreasing rate of pipemidic acid in urine for male and female were consistent, respectively. J. Clin. Lab. Anal. 24:327–333, 2010.

Key words: electrochemiluminescence; capillary electrophoresis; proline; pipemidic acid; urine

INTRODUCTION

Proline (Pro) is commonly found in connective tissue proteins such as collagen at high concentrations. Pro presents in biological fluids in free-, peptide- and protein-form and the contents of Pro vary in association with various diseases such as bone diseases, tumors and chronic uremia (1). Pipemidic acid (PPA) is a synthetic antimicrobial drug. This compound was extensively used in the treatment of Gram-negative urinary tract infections, but if the administration dosage were too high, it would cause hematuria, and also severely damages DNA in the absence of an exogenous metabolizing system (2). Quality control of PPA dosage and its monitoring in biological fluids by quick automated techniques is an important analytical task.

Several methods have been reported for the determination of Pro by spectrophotometry (3), mass spectrometry (4), chemiluminescence (CL) (5), liquid chromatography (LC) (1) and capillary electrophoresis (CE) (6). Several analytical techniques have been reported for the determination of PPA, including spectrophotometry (7,8), fluorimetry (9,10), LC (11,12), CL (13–15) and EC (16,17). However, these methods require more sophisticated instrumentation or are more time-consuming. Thus, it is very important to

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develop a simple and rapid method for simultaneous detection of Pro and PPA.

The CE has been widely used in the clinical laboratory (18). Separations with several hundred thousand theoretical plates have been achieved even with simple capillary zone electrophoresis in its early day (19). The high efficiency, powerful resolution, fast separation, low instrumental cost and low consumption of samples and reagents are the main advantages of CE over high performance liquid chromatography. The most commonly used detection modes available for CE were fluorescence detection, laser-induced fluorescence detection (20,21), UV-visible spectrophotometric detection (22,23), mass spectrometry (24) and CL (25). The development of electrochemiluminescence (ECL) detection for CE was critically reviewed (26). ECL detection, in comparison with other modes, offered lower background noise, higher detection sensitivity and requires simple and inexpensive instrumentation.

The CE coupling with Tris(2,2′-bipydidyl)Ruthenium (II) ($Ru(bpy)_3^{2+}$) CL detection has been applied for the determination of aliphatic amines (27), β-blockers (28), ascorbic and dehydroascorbic acids in orange fruit juice (29), procyclidine in human urine (30), diphenhydramine in rabbit plasma and urine samples (31), lincomycin in urine (32) and illicit drugs on banknotes (33). There is no reported method for separation and simultaneous determination of PPA and Pro till now.

The aim of this study is to develop an efficient method for simultaneous determination of Pro and PPA 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 Pro and PPA in urine samples with satisfactory results.

EXPERIMENTAL

Reagents and Chemicals

Pro was obtained from Sigma (St. Louis, MO). Tris(2,2′-bipydidyl)ruthenium(II) chloride hexahydrate (TBR) was purchased from Aldrich Chemical Co. (Milwaukee, WI). PPA was obtained from Institute of Medical Biotechnology (Beijing, China). Standard stock solutions of Pro and PPA were prepared with double-distilled water and stored at 4°C in a refrigerator. Working standard solutions were prepared by dilution of standard stock solutions with double-distilled water. Phosphate buffer used in the detection cell and as electrophoresis running buffer was prepared by using equimolar (5–80 mM) amount of disodium hydrogen phosphate and sodium dihydrogen phosphate. The appropriate pH (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 include 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. Separation voltage was set at 15 kV. The reservoir, solution of 5 mM $Ru(bpy)_3^{2+}$ and 50 mM phosphate buffer were replaced once for 4 hr. 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 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 was set at 800 V for collecting ECL signal. The sample solution was injected by electromotion for 10 s at 10 kV. Each sample was performed in triplicate.

Sample Analysis

After the healthy volunteer was treated with oral administration of PPA, the urine sample was collected at different times. A 1-mL volume of urine sample was diluted with 10 mL of double-distilled water. A 0.5-mL volume 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 min at 4,000 rpm. The centrifugate was used for CE–ECL analysis. Under the optimized conditions, ECL detection at 1.15 V, 20 mM phosphate buffer at pH 9.6, 5 mM $Ru(bpy)_3^{2+}$ and 70 mM separation buffer at pH 8.0, the calibration curves were generated with the standard solutions of 40, 50, 60, 70, 80 and 90 $\mu g\,mL^{-1}$ of Pro and 2, 4, 6, 8 and 10 $\mu g\,mL^{-1}$ of PPA. Under same conditions, the content of Pro and PPA in the urine sample collected at different times was determined.

RESULTS AND DISCUSSION

Optimization of Separation Conditions

Effect of separation buffer pH

The separation buffer pH influences not only the net 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. Pro and PPA were completely separated ($Rs > 6$), when the separation buffer pH from 5.0 to 11.5 was used. The resolution (Rs) between Pro and PPA was calculated by 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 PPA, respectively, W_{b1} and W_{b2} are the peak widths of Pro and PPA measured at the baseline. The ECL intensity of Pro and PPA was observed when the separation buffer pH was in the range of 5.0–11.5 (Fig. 1). The effect of the buffer pH on the ECL intensity of Pro and PPA was slight. The reason could be ascribed to the buffer capacity of the detection cell in which the pH environment did not change even when small volume of separation buffer at different pH value was introduced through the capillary. 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.

Effect of separation buffer concentration

The effect of the concentration of separation buffer (5–40 mM) on ECL intensity and the separation of Pro and PPA was tested using a pH of 9.6 in the buffer solution, as shown in Figure 2. 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, there was insufficient buffer capacity. The concentration at 20 mM was found to be the optimal concentration for the separation of Pro and PPA in CE–ECL system.

Effect of separation voltage

In CE/ECL system, the detection reservoir used is the same as previously reported (34). The influence of separation voltage on the emission intensity was investigated from 5 to 25 kV. The ECL intensity increased as the separation voltage increased up to 15 kV, where it plateaued. The electroosmosis flow should increase with increasing separation voltage, thus more analyte in the effluent arrives in the diffusion layer of working electrode within a given time, higher ECL signal could 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 light producing reaction. These two factors offset each other when separation voltage ranged from 15 to 25 kV, so ECL signal reached a plateau (34). A 15 kV was chosen as a separation voltage in our experiment to ensure high ECL intensity and good reproducibility.

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

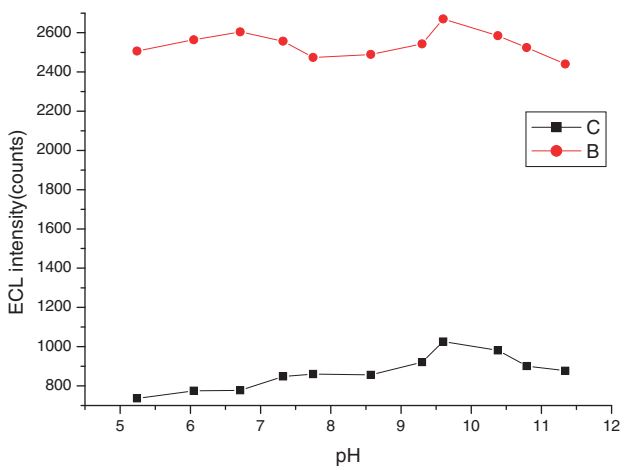

Fig. 1. Effect of pH value of the separation buffer in capillary on ECL intensity. B: Pro, 50 μg mL^{-1}; C: PPA, 20 μg mL^{-1}; $Ru(bpy)_3^{2+}$, 5 mM; phosphate buffer, 70 mM, pH = 8.0; electrokinetic injection, 10 s at 10 kV; separation buffer, 10 mM; separation voltage, 15 kV.

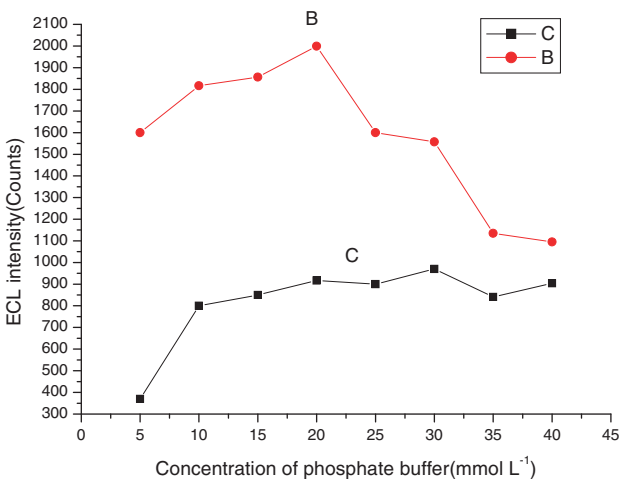

Fig. 2. Effect of concentration of the separation buffer in capillary on ECL intensity. B: Pro, 50 μg mL^{-1}; C: PPA, 20 μg mL^{-1}; separation buffer, pH = 9.6.

applied potential on the analyte ECL signals was tested by changing the potential from+1.00 to+1.25 V. As shown in Figure 3, the highest ECL intensity was at 1.15 V, as shown in the hydrodynamic voltammograms, hence, the most sensitive detection potential for Pro and PPA was 1.15 V.

Effect of the phosphate buffer pH on detection

The effect of pH on the ECL intensity was investigated in the pH range of 4.5–11. The result is shown in Figure 4.

ECL intensity of the sample solutions were increased dramatically with increasing pH value of the buffer from 5.5 to 8.0, and remained steady when the buffer pH ranged from 8.0 to 10. When the buffer pH exceeded 10, the CL response decreased. The role of the OH^- ion in the reaction mechanism of the two analytes appeared to be crucial. The reason for the decrease in emitted light above pH 10 can be ascribed to the reduced availability of $Ru(bpy)_3^{3+}$ owing to the competitive reaction with the OH^- ion, which presents considerable concentration levels at high pHs (29). Therefore, the buffer pH value was set at 8.0 in this study.

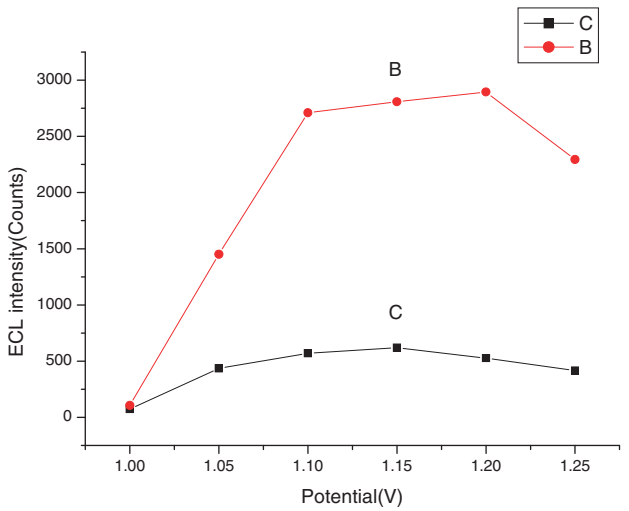

Fig. 3. Effect of detection potential on ECL intensity. B: Pro, 50 μg mL^{-1}; C: PPA, 20 μg mL^{-1}; $Ru(bpy)_3^{2+}$, 5 mM; phosphate buffer, 50 mM; separation buffer, 10 mM, pH = 8.0.

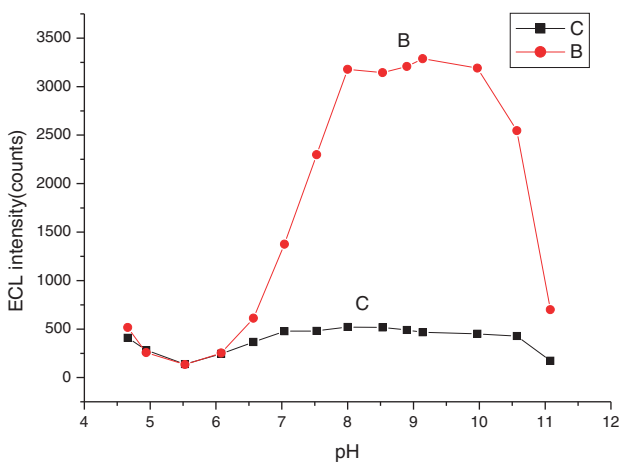

Fig. 4. Effect of pH value of the phosphate buffer in ECL cell on ECL intensity. B: Pro, 50 μg mL^{-1}; C: PPA, 20 μg mL^{-1}; phosphate buffer, 50 mM.

Effect of the phosphate buffer concentration on detection

Another investigation of the phosphate buffer concentration from 20 to 80 mM (pH 8.0) in the detection cell was also performed, as shown in Figure 5.

The highest ECL intensity of Pro and PPA was obtained when the concentration of the buffer was 70 mM. If the ionic strength of background electrolyte were 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 70 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, this result may be owing to the effect of increased electrophoretic current on the ECL detector. The concentration of the phosphate buffer was set at 70 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

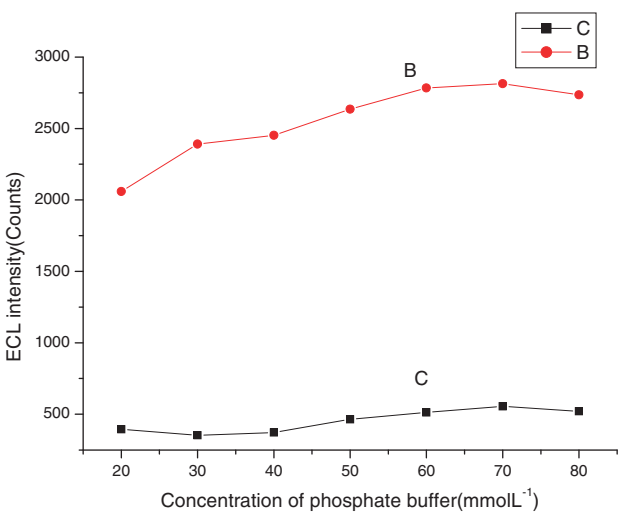

Fig. 5. Effect of concentration of the phosphate buffer in ECL cell on ECL intensity. B: Pro, 50 μg mL^{-1}; C: PPA, 20 μg mL^{-1}; phosphate buffer, pH = 8.0.

parameters. The reaction rate is a function of the concentrations of species involved in the reaction. High sensitivity is obtained with increasing the concentration of $Ru(bpy)_3^{2+}$, at the same time the background noise increased and larger amount of expensive reagent ($Ru(bpy)_3Cl_2 \cdot 6H_20$) was consumed. A low concentration of $Ru(bpy)_3^{2+}$ leads to a lower background noise. Figure 6 showed the effect of the concentration of $Ru(bpy)_3^{2+}$ on the ECL intensity of Pro and PPA. To obtain a higher S/N value, ECL efficiency and a moderate reagent consumption, 5 mM $Ru(bpy)_3^{2+}$ was used in our experiment. After operating for 3–4 hr, the $Ru(bpy)_3^{2+}$ solution was replenished in order to maintain reproducibility.

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

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^-(\text{anode})$$

$$Ru(bpy)_3^{3+} + \text{Pro} \rightarrow Ru(bpy)_3^{+} + \text{oxidized Pro}$$

$$Ru(bpy)_3^{3+} + \text{PPA} \rightarrow Ru(bpy)_3^{+} + \text{oxidized PPA}$$

$$Ru(bpy)_3^{+} + Ru(bpy)_3^{3+} \rightarrow Ru(bpy)_3^{2+*} + Ru(bpy)_3^{2+}$$

$$Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + e^-(\text{anode}) + h\nu(620\,\text{nm})$$

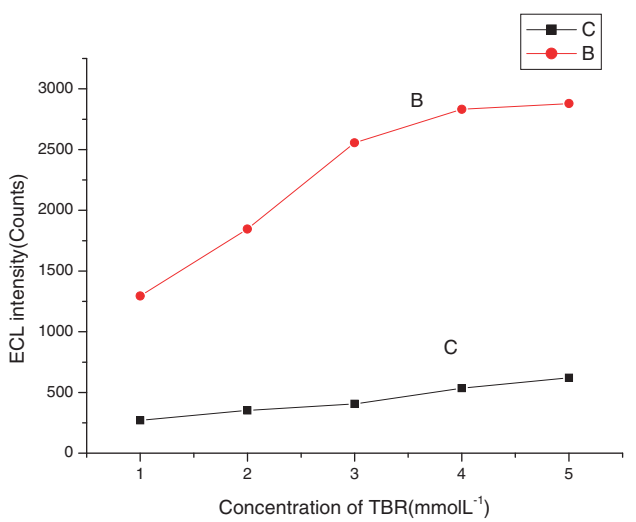

Fig. 6. Effect of concentration of TBR on ECL intensity. B: Pro, 50 μg mL^{-1}; C: PPA, 20 μg mL^{-1}; phosphate buffer, 70 mM, pH = 8.0.

Reproducibility, Linearity and Detection Limit

Under optimized conditions, a standard mixture solution containing 6 μg mL^{-1} of Pro and PPA was injected consecutively 11 times to determine the reproducibility of ECL intensity based on peak height and migration time. Relative standard derivations for the ECL intensity and the migration time were 3.2 and 0.9% for Pro, and 3.7 and 1.2% for PPA, respectively. The high reproducibility indicates that this approach is accurate for detection of Pro and PPA.

To investigate the detection linearity of Pro and PPA, a series of standard mixture solutions containing the two species were tested. The standard curves were linear in the range of 0.1–90 μg mL^{-1} for Pro and 0.4–100 μg mL^{-1} for PPA. The calibration equations and regression coefficients were $y = 132.8x + 24.9$ and $R = 0.998$ for Pro, $y = 29.7x + 129.2$ and $R = 0.997$ for PPA in terms of peak height response as a function of analyte concentration.

The limit of detection was determined as the sample concentration that produces a peak with a height three times the level of the baseline noise. Detection limit (3σ) of the proposed method was 0.02 μg mL^{-1} for Pro and 0.06 μg mL^{-1} for PPA. Compared with traditional CE method, the present method displayed good performance with sensitivity, selectivity, simplicity and rapidity.

Application to Human Urine

The proposed CE–ECL method was applied to the determination of Pro and PPA in human urine under the optimized conditions. The healthy volunteer was treated simultaneously with an oral administration of 500 mg PPA capsule. The urine samples were collected at 4, 8, 12, 24 hr, respectively, after oral administration of PPA for determination of PPA and Pro. The urine collected before dosing was employed as a blank for determination of PPA. All urine samples were treated as shown in "Sample Analysis" section in "Experimental" and examined with CE–ECL system. Electropherograms of standard and urine sample of Pro and PPA are shown in Figure 7. The content of Pro and PPA in the urine samples was determined. After each determination, a 50 μg mL^{-1} standard solution of Pro and PPA was added in the urine sample. The fortified urine samples were analyzed. The content and the recovery for Pro and PPA are summarized in Table 1, along with the relative standard deviations (RSDs). The recoveries were 95.2–98.6% for Pro and 94.4–97.1% for PPA, and the RSDs were 3.8–4.7% for Pro and 4.1–5.1% for PPA. The result shows the content of Pro in urine for male is higher than that for female, and the content of PPA in urine for male and female is consistent. Otherwise, during 4–24 hr the decreasing rate of Pro in urine is 3.3% for male and 4.5% for female, and the decreasing

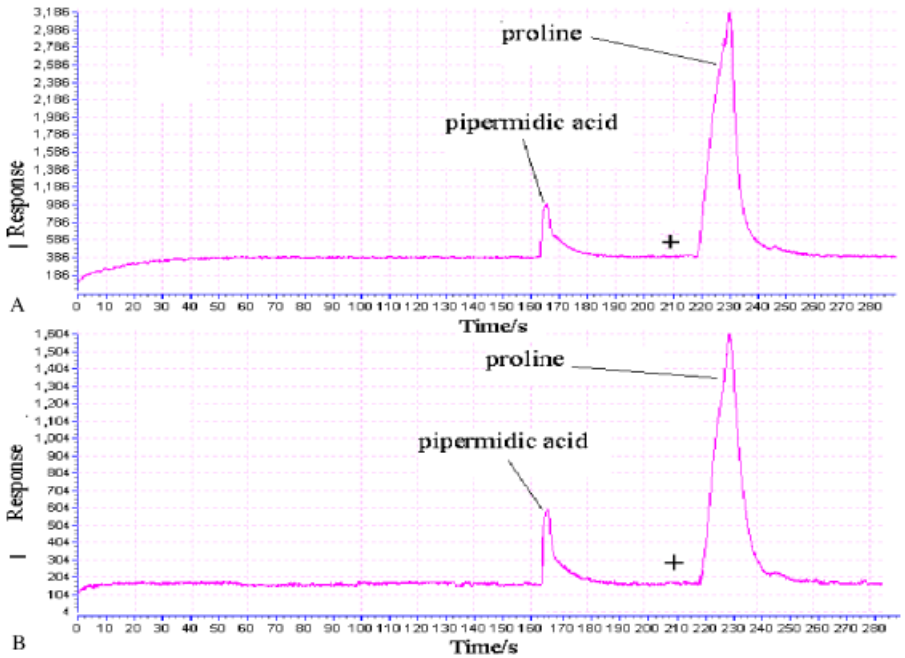

Fig. 7. Electropherograms of the standard solution (**A**) and human urine (**B**) $Ru(bpy)_3^{2+}$, 5 mM; phosphate buffer, 70 mM, pH = 8.0; electrokinetic injection, 10 s at 10 kV; separation buffer, 20 mM, pH = 9.6; separation voltage, 15 kV.

TABLE 1. Determination Results and Recovery of Pro and PPA for Urine Samples

Analyte	Sample	Time (hr)	Content ($\mu g\,mL^{-1}$)	Added ($\mu g\,mL^{-1}$)	Found ($\mu g\,mL^{-1}$)	Recovery (%)	RSD ($n = 5$) (%)
Pro	Male	4	864.1	50	913.4	98.6	4.2
	Female	4	801.3	50	850.2	97.8	4.5
	Male	8	824.3	50	872.4	96.2	3.9
	Female	8	754.9	50	802.5	95.2	4.0
	Male	12	812.7	50	861.3	97.2	4.3
	Female	12	734.1	50	783.0	97.8	4.7
	Male	24	835.2	50	883.5	96.6	4.4
	Female	24	765.4	50	814.5	98.2	3.8
PPA	Male	4	69.4	50	117.3	95.8	4.6
	Female	4	68.3	50	115.8	95.0	4.4
	Male	8	53.2	50	100.4	94.4	4.9
	Female	8	57.1	50	105.5	96.8	4.1
	Male	12	39.7	50	88.2	97.1	5.1
	Female	12	41.9	50	89.7	95.7	4.8
	Male	24	29.4	50	76.8	94.8	5.0
	Female	24	28.9	50	77.1	96.4	4.2

rate of PPA in urine for male and female is 57.6 and 57.7%, respectively.

CONCLUSION

A new method has been developed to determine Pro and PPA in urine. Under optimized conditions, the approach of CE–ECL with $Ru(bpy)_3^{2+}$ showed good performance in terms of selectivity, sensitivity, repeatability, short analysis time and linearity. The validated method can be used to routine determination of Pro and PPA in urine and clinical study.

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