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Determination of enoxacin and ofloxacin by capillary electrophoresis with electrochemiluminescence detection in biofluids and drugs and its application to pharmacokinetics

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ABSTRACT: A novel and sensitive method for the simultaneous determination of enoxacin and ofloxacin has been established using capillary electrophoresis (CE) coupled with electrochemiluminescence (ECL) detection based on the ECL enhancement of tri(2,2-bipyridyl)ruthenium(II). The conditions for sample solvent type, CE separation and ECL detection were investigated systematically. The analytes were well separated and detected within 7 min. The limits of detection (S/N = 3) of enoxacin and ofloxacin are 9.0×10^{-9} and 1.6×10^{-8} mol/L, respectively. The precisions (RSD%) of intraday and interday are less than 2.1 and 4.0%, respectively. The limits of quantitation (S/N = 10) of enoxacin and ofloxacin are 3.2×10^{-7} and 5.4×10^{-7} mol/L in human urine samples and 4.1×10^{-7} and 6.9×10^{-7} mol/L in human serum samples, respectively. The recoveries of enoxacin and ofloxacin at different concentration levels in human urine, serum and eye drop samples are between 94.0 and 106.7%. The proposed method was successfully applied to the determination of the enoxacin and ofloxacin in human urine, serum and eye drop samples and the monitoring of pharmacokinetics of ofloxacin in human body. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: capillary electrophoresis; electrochemiluminescence; enoxacin; ofloxacin; pharmacokinetics

Introduction

Electrochemiluminescence (ECL) detection is a special chemiluminescence (CL) where CL emission is generated in the process of substance oxidation and reduction at an electrode surface. ECL is undergoing rapid development and has numerous promising analytical applications because it can reduce interferences from solution impurities and eliminate the need for complicated construction of postcolumn reactors, and thus offers low background noise and high detection sensitivity (Qiu *et al.*, 2003). Tri(2,2-bipyridyl)ruthenium(II), namely $Ru(bpy)_3^{2+}$, is the most efficient and widely used ECL reagent due to high luminescence efficiency and stability in aqueous media. The light signal accompanies the oxidation of $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$ on the electrode surface and then react with tertiary, secondary or primary alkyl amines (Gorman *et al.*, 2006; Liu *et al.*, 2009b). $Ru(bpy)_3^{2+}$ ECL has been extensively utilized as a detection means for many amine-containing analytes in HPLC and flow injection analysis. CE is a useful separation technique because of its high resolution, short analysis time, small sample volume and low operational cost (Richter, 2004). Recently, CE-CL has been widely developed and become a useful tool for rapid detection of various analytes such as metal ions (Ren and Huang, 2001), amino acids (Zhao *et al.*, 2005), proteins (Zhi *et al.*, 2007) and antigen–antibody complexes (Wang and Ren, 2005). The marriage of CE to ECL is a sensitive and efficient analytical technique and has got excellent performance for the analysis of proteins (Li *et al.*, 2007; Guo *et al.*, 2009), amino acids (Li *et al.*, 2006) and drugs (Zhao *et al.*, 2004; Yuan *et al.*, 2009; Liu *et al.*, 2008a, 2009a, b; Xu *et al.*, 2006; Deng *et al.*, 2008).

Enoxacin (EN) and ofloxacin (OF) are fluoroquinolones (FQs) which comprise an important group of broad-spectrum synthetic antimicrobial agents in human and veterinary medicine. They inhibit bacterial DNA-gyrase in cells so that the bacteria cannot reproduce and eventually die (Yoke and Froc, 2000). These compounds have demonstrated activities against Gram-positive and Gram-negative bacteria, so they have been broadly applied in the clinical treatment of certain infections, such as prostate, skin, pulmonary, digestive and urinary tract infections, because of good absorption and low frequency of adverse effect (Christodoulou *et al.*, 2007). In addition, they are also used for prophylaxis and as feed additives for mass gain promotion (Mitani and Kataoka, 2006). However, their overfull use also

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Abbreviations used: CL, chemiluminescence; EN, enoxacin; FQ, fluoroquinolones; OF, ofloxacin.

caused serious pollution of FQs derivative in biology and circumstance. Therefore, it has become more and more important to develop sensitive and rapid method for the determination of the content of FQs antibiotics in biochemical analysis. Recently, HPLC/LC (Christodoulou *et al.*, 2007; Xiao *et al.*, 2008; Espinosa-Mansilla *et al.*, 2005; Cañada-Cañada *et al.*, 2007), flow injection analysis (Chen *et al.*, 2008; Zhang *et al.*, 2005; Rao *et al.*, 2000) and CE (Lü *et al.*, 2005; Horstkötter and Blaschke, 2001; McCourt *et al.*, 2003; Liu *et al.*, 2008b; Lin *et al.*, 2004; Fan *et al.*, 2007; Yin *et al.*, 2004) have been used in the determination of enoxacin and/or ofloxacin. In CE methods, the detection modes used mainly included UV–vis (Lü *et al.*, 2005), laser-induced fluorescence (Horstkötter *et al.*, 2001), mass spectrometry (McCourt *et al.*, 2003), CL (Liu *et al.*, 2008b), photodiode array detection (Lin *et al.*, 2004) and potential gradient detection (Fan *et al.*, 2007). Yin *et al.* (2004) determined ofloxacin by short-CE-ECL and the limits of detection (S/N = 3) of ofloxacin was 5.0×10^{-7} mol/L. However, CE-ECL method for the analysis of EN and OF, as well as its extended application in pharmacokeinetics has not been reported so far.

In this work, a sensitive method was developed for the simultaneous determination of EN and OF with CE-ECL. The conditions for sample solvent type, CE separation and ECL detection were investigated in detail. The developed method was applied to the determination of EN and OF in human urine, sera and eye drop samples and the monitoring of pharmacokinetics of OF in human body.

Experimental

Chemicals and Materials

EN and OF were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The tablets of OF were acquired from Henan Tianfang Pharmaceutical Co. Ltd (Zhumadian, China). EN eye drops were acquired from Nanjing Tianlang Pharmaceutical Co. Ltd (Nanjing, China). OF eye drops were acquired from Zhengzhou Zhuofeng Pharmaceutical Factory (Zhengzhou, China). Tris(2,2′-bipyridyl) ruthenium (II) chloride hexahydrate was purchased from Alfa Aesar (A Johnson Matthey Company, Ward Hill, MA, USA). All chemicals and reagents were of analytical grade and used without further purification.

Stock solutions, 5 mmol/L, of EN and OF were prepared by dissolving the standard sample in 0.1 mol/L HCl and stored in dark in the refrigerator. The pure water (18.2 MΩ cm) was processed with an Ultrapure Water System (Kangning Water Treatment Solution Provider, China). A series of working standard solutions were used by diluting the stock solution with acetonitrile (MeCN)–water (7:3, v/v) mixtures for calibration. All other solutions were prepared with pure water. They were stored in the refrigerator at 4°C and filtered through 0.22 μm cellulose acetate filters (Shanghai Xingya Purification Material Factory) before use.

CE-ECL Apparatus

The CE-ECL experiments were performed on a model MPI-B capillary electrophoresis electrochemiluminescence system (Xi'an Remax Electronic Science-Tech Co. Ltd, Xi'an, China). The system provided a programmable high-voltage power supply (0–20 kV), an electrochemical potentiostat, a multifunction CL detector and a multichannel data collection analyzer. Output ECL intensity was amplified and recorded in a computer using the MPI-B software.

The end-column ECL cell was composed of a 500 μm Pt disk working electrode, an Ag/AgCl reference electrode (KCl saturated), and a Pt wire counter electrode. The surface of the working electrode was polished sequentially with 0.3 and 0.05 μm α-Al_2O_3 on a piece of polishing cloth until a mirror-smooth surface appeared and then was sonicated for 10 min in water. The electrode was subjected to repeated cycling in the potential region of 0.2–1.25 V (vs Ag/AgCl) to obtain a reproducible cyclic voltammogram before each experiment. In order to obtain good precisions in analysis, about 300 μL of $Ru(bpy)_3^{2+}$ solution was added into the detection cell before analysis.

Procedure

All separations were performed in a 55 cm long fused-silica capillary with 50 μm i.d. and 375 μm o.d. (Yongnian Reafine Chromatography Ltd, Hebei, China). The new capillary was rinsed sequentially with 2.0 mol/L CH_3OH-NaOH (2 g NaOH dissolved in 25 mL 4:1/CH_3OH:H_2O, v/v), 1.0 mol/L NaOH, 1.0 mol/L HCl, H_2O and electrophoretic buffer for 20 min. At the beginning of each day, the capillary was flushed with 0.1 mol/L NaOH, 0.1 mol/L HCl and H_2O and equilibrated with the electrophoretic buffer for 10 min successively so as to maintain an active and reproducible inner surface. The capillary was rinsed sequentially with 0.1 mol/L NaOH, 0.1 mol/L HCl, H_2O and electrophoretic buffer for 2 min after every five runs. The voltage of photomultiplier tube (type CR105, Beijing Binsong Photonics, China) for collecting the ECL signal was set at –850 V in the process of detection. The inlet end of the capillary was held at a positive potential and the outlet end was maintained at ground. $Ru(bpy)_3^{2+}$, 5 mmol/L, prepared in 50 mmol/L phosphate buffer solution (PBS) was added in the detection cell. The peak area was used for the analysis.

The Preparation of Urine and Serum Samples

Fresh human urine samples of two healthy female volunteers were acquired from Xinyang Normal University. OF (300 mg) was orally administered to the volunteers in a pharmacokinetics study. About 5 mL urine samples were collected at 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12 h after oral administration of 300 mg OF. The two volunteers were asked to drink sufficient and comparable amounts of water throughout the collection period. Blank urine samples were also collected just before oral administration of OF for the preparation of spiked samples, limit of quantitation (LOQ) and calibration curve. Samples of 1.2 mL urine (or spiked urine samples) were pipetted into clean 5 mL centrifugation tubes. Then 2.8 mL MeCN was added to each sample and the tubes were capped and shaken for 5 min. The samples were centrifuged at 3000 rpm for 10 min to remove deposits. The supernatant was taken out and then analyzed according to the above-mentioned description. The each sample was analyzed immediately after preparation procedure and special care had to be taken to keep the samples away from the light.

The healthy human blood sample was acquired from Xinyang Central Hospital (Xinyang, China). The blood was centrifuged at 3000 rpm for 10 min to acquire serum and the serum was stored in the refrigerator at 4°C before use. A series of serum or spiked serum samples were treated MeCN–water (7:3, v/v) mixtures and shaken for 5 min, then the mixtures were centrifuged at 3000 rpm for 10 min to remove deposit. The supernatant was taken out and then analyzed immediately.

Results and Discussion

Enhanced ECL of $Ru(bpy)_3^{2+}$ by Ofloxacin

EN and OF have similar molecular structures and contain tertiary amine groups. Therefore, we take OF as a model analyte to study the ECL performances of FQs in this work. The ECL behavior and cyclic voltammograms of OF are shown in Fig. 1A and B, respectively. Under cyclic voltammetric conditions, the rise of ECL intensity began at 1.00 V and increased significantly with the increase of potential. In a comparison, the ECL intensity was increased remarkably as OF was added in the $Ru(bpy)_3^{2+}$ solution. These observations indicated that OF can react with the ruthenium species in the ECL process, and it can enhance the emitted light

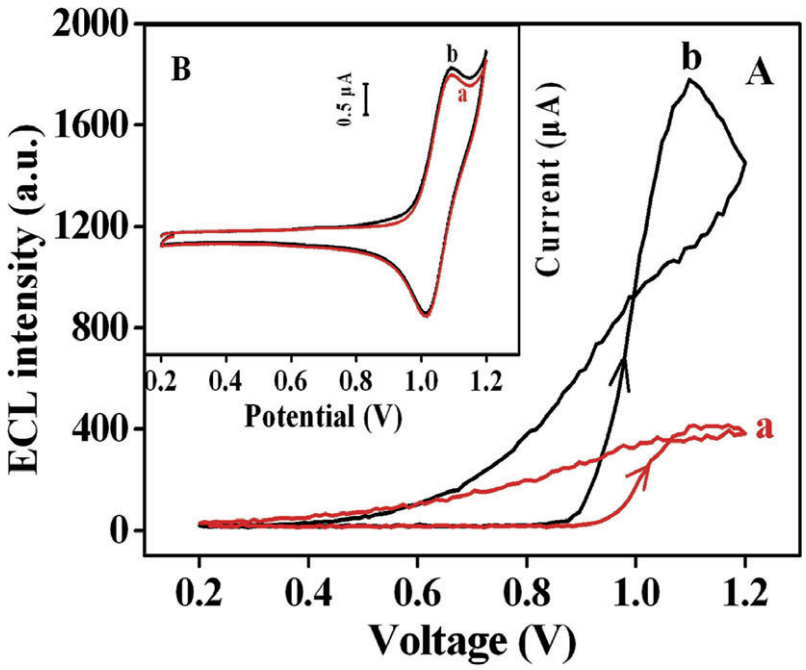

Figure 1. (A) Dependence of the ECL intensity on applied voltage curve of 5 mmol/L $Ru(bpy)_3^{2+}$ without (a) and with (b) 2×10^{-5} mol/L OF. Condition: 50 mmol/L PBS at pH 8.0 in the detection cell. (B) Cyclic voltammogram of 5 mmol/L $Ru(bpy)_3^{2+}$ without (a) and with OF (b) in 50 mmol/L PBS (pH 8.0) at a scan rate of 100 mV/s.

intensity. According to the earlier reports (Miao, 2008), the possible mechanism for the enhanced ECL emission might occur in the steps below:

$$Ru(bpy)_3^{2+} - e^- \rightarrow Ru(bpy)_3^{3+} \quad (1)$$

$$OF - e^- \rightarrow OF^{\bullet +} \quad (2)$$

$$OF^{\bullet +} \rightarrow OF^{\bullet} + H^+ \quad (3)$$

$$Ru(bpy)_3^{3+} + OF^{\bullet} \rightarrow Ru(bpy)_3^{2+*} + product \quad (4)$$

$$Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + h\nu \quad (5)$$

Choice of Sample Solvents

In this work, pure water, 0.1 mol/L NaOH and 0.1 mol/L HCl were investigated as sample solvents to prepare the stock solution of EN and OF, respectively. The results showed that the signal intensity of prepared stock solution in 0.1 mol/L HCl yielded a stronger signal than in water and 0.1 mol/L NaOH. Addition of organic compounds like MeCN can lower the sample conductivity, which can induce a sample stacking phenomenon in CE electrokinetic injection mode (Shihabi, 2000). It has also been reported to be effective in removing proteins in biological fluid samples prior to CE experiments. To optimize the analytical sensitivity and separation efficiency, pure water, 0.1 mol/L HCl, electrophoretic buffer and MeCN–water solution were used to dilute the stock solution as solvents, respectively. The results indicated that using MeCN–water solution as the solvent could improve both sensitivity and resolution markedly. The effects of the ratio of MeCN to water were further examined and the results are shown in Fig. 2. It can be seen that ECL intensity increased with increase in ratio of MeCN. The resolution (*Rs*) was calculated with the following equation: $Rs = 2(t_2 - t_1)/(W_{b1} + W_{b2})$, where t_1 and t_2 are the migration times of two adjacent analytes. W_{b1} and W_{b2} stand for peak

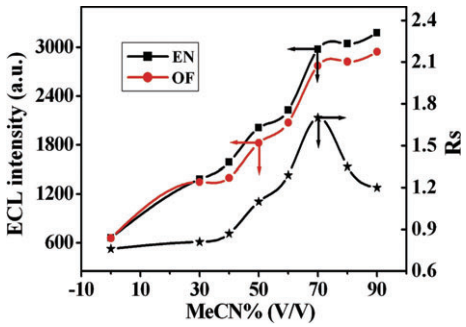

Figure 2. Effect of ratio of MeCN to water on ECL intensity. Conditions: electrophoretic buffer, 15 mmol/L phosphate buffer at pH 9.0; electrokinetic injection, 10 kV × 10 s; separation voltage, 15 kV; ECL solution, 5 mmol/L $Ru(bpy)_3^{2+}$ with 50 mmol/L PBS at pH 8.0. 3 μmol/L EN, 5 μmol/L OF.

widths of two adjacent analytes measured at the baseline. The *Rs* between EN and OF also greatly improved when the ratio of MeCN to water reached 70%, and then dropped as the ratio was further increased. A ratio of 70% (v/v) MeCN in water was selected as the optimal standard solution diluting solvent in the following experiments.

Effect of Detection Potential

ECL intensity is dependent on the rate of the light-emitting chemical reaction that is otherwise determined by the potential applied to the electrode (Haapakka *et al.*, 1982). Therefore, the influences of the applied potential from 1.05 to 1.35 V on ECL intensity were studied and the experimental results are shown in Fig. 3. An increase in the applied potential from 1.05 to 1.15 V resulted in an enhancement in the ECL intensity while further

increase in the potential, on the contrary, reduced the ECL intensity. The results agree well with those found in the CV experiments described above. Hence, 1.15 V was selected.

Effect of Buffer pH in Detection Reservoir

The ECL reaction of $Ru(bpy)_3^{2+}$ with alkylamine well known as a pH-dependent process and the maximum emission has been observed under slightly basic conditions (Brune and Bobbitt, 1991). The relationship between the ECL intensity and pH value in the detection reservoir from 7.0 to 9.0 was investigated. With the increase in solution pH, the ECL intensity first increased and reached its apex at pH 8.0, then decreased gradually. Therefore, the buffer pH in the detection reservoir was fixed at 8.0.

Effects of pH and Concentration of Electrophoretic Buffer

The pH and concentration of electrophoretic buffer can influence the ECL intensity of analytes. The effects of PBS electrophoretic buffer pH on ECL intensity were examined in the range of 8.1–9.9. The results indicated that the highest ECL intensities of EN and OF were achieved at pH 9.0. When the pH was above 9.0, the ECL responses decreased. As a result, pH 9.0 was used. The effects of concentration of electrophoretic buffer from 9 to 24 mmol/L were also investigated and it was found that higher ECL intensity was acquired as the buffer concentration was 15 mmol/L. Thus, 15 mmol/L was employed.

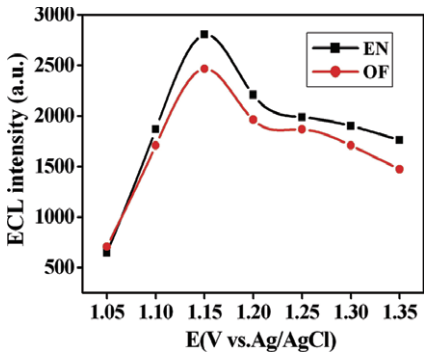

Figure 3. Effect of detection potential on ECL intensity. Conditions are the same as in Fig. 2.

Effect of Separation Voltage

Figure 4 shows a plot of ECL intensity and *Rs* as a function of separation voltage from 9 to 19 kV. It can be seen from Fig. 4 that the ECL intensity increased with the separation voltage increase from 9 to 15 kV, and then it reduced as the voltage was further increased. *Rs* between EN and OF reached a maximum value at separation voltage 12 kV and then decreased; however, Rs still could be kept at 2.0 (>1.5) when the separation voltage was 13 kV. As a compromise choice, 15 kV was used in the following separations.

Analytical Performance

Under the optimal conditions [detection potential, 1.15 V; electrokinetic injection, 10 kV for 10 s; separation voltage, 15 kV; electrophoretic buffer, 15 mmol/L PBS at pH 9.0; 5 mmol/L $Ru(bpy)_3^{2+}$ with 50 mmol/L PBS at pH 8.0 in the detection cell], EN and OF were baseline resolved within 7 min and a typical electropherogram is shown in Fig. 5. To evaluate the linearity of the established method, the calibration curves were made by plotting the peak area values against the analyte concentrations. It can be seen from Table 1 that the regression coefficients of the calibration curves are greater than 0.9973. LOD was considered the minimum analyte concentration, yielding an S/N ratio equal to 3.

The precision (RSD%, $n = 5$) of the peak area of a mixture of 3.0×10^{-6} mol/L EN and 5.0×10^{-6} mol/L OF were 1.5 and 2.1% within a day, and 4.0 and 2.5% in 3 days, respectively. The RSDs of the migration time of EN and OF were 1.1 and 1.3% within a day, and 1.9 and 1.7 % in 3 days, respectively.

Applications

To evaluate the applicability of the present CE-ECL method to real samples, it was used to determine EN and OF in human urine,

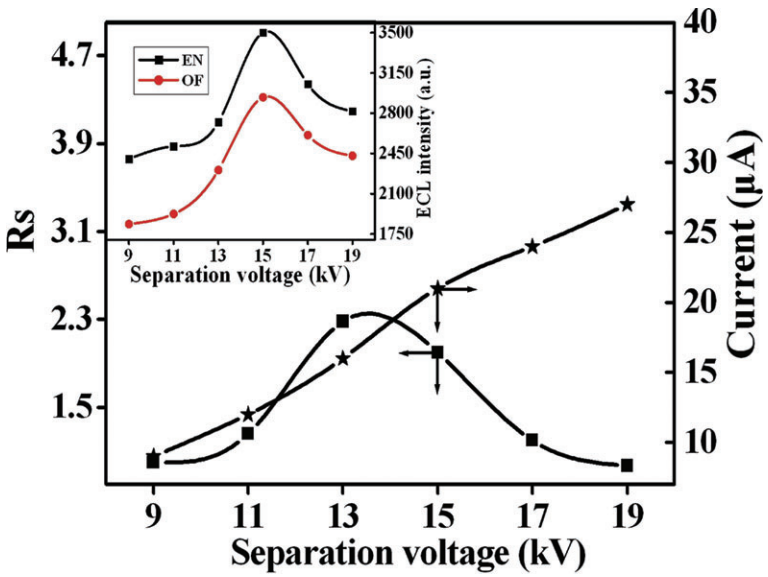

Figure 4. Effect of separation voltage on ECL intensity. Other conditions are the same as in Fig. 3.

human serum and eye drop samples. The typical electropherograms of blank urine sample of healthy person and urine sample spiked with 1.0×10^{-5} mol/L EN and 1.5×10^{-5} mol/L OF are illustrated in Fig. 6A and B, respectively. The results indicate that the two analytes were not detected in urine samples of healthy person but could be seen and resolved in urine samples spiked with standard substances. In addition, there were also several unknown peaks from the unknown compoundsin the urine sample matrix. In urine analysis, the recoveries of the two analytes at three different spiked concentration levels were carried out and ranged from 97.6 to 104.0% for EN and from 95.4 to 105.0% for OF (listed in Table 2). The RSDs of peak area were less than 4.7%. The LOQs (defined as the lowest analyte concentration at S/N = 10) of EN and OF in urine samples were 3.2×10^{-7} and 5.4×10^{-7} mol/L, respectively. In serum analysis, the recoveries of two analytes at three different spiked concentration levels were carried out and ranged from 96.0 to 101.9% for EN and from 94.0 to 105.0% for OF (listed in Table 2). The RSDs of peak areas were less than 4.6%. The LOQs of EN and OF in serum samples were 4.1×10^{-7} and 6.9×10^{-7} mol/L, respectively.

The proposed method was also used in the analysis of the EN and OF in eye drop samples. It can be seen from Table 3 that the measured contents of the analytes in eye drop samples were very close to the labeled values. In addition, the recoveries of EN and OF at two different spiked concentration levels were in the range 97.7–101.3% for EN and 100.9–106.7% for OF, respectively. The RSDs of peak area and migration times in eye drop analysis were less than 3.2 and 1.2%, respectively. The LOQs of EN and OF in eye drop samples were 5.8×10^{-8} and 1.1×10^{-7} mol/L, respectively. These results indicate that the proposed method is accurate and reliable.

In the study of the OF pharmacokinetics, two healthy female volunteers received an oral administration of 300 mg of OF tablets. The urine samples were collected and analyzed immediately before the oral dose and again at 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12 h post-dose. The results of the two volunteers were plotted as concentration–time profiles. As shown in Fig. 7, the maximal content of OF in the urine samples of volunteers was achieved in 4 h after the oral dose and then the OF concentration was observed to decrease. The results showed that the OF content of volunteer 2 is higher than that of volunteer 1 at any time after oral administration. This might be caused by the actual metabolizability diversity between individuals.

Conclusion

A novel and sensitive CE-ECL method was proposed for the simultaneous determination of EN and OF in human urine, serum and eye drop samples and the monitoring of urine pharmacokinetics of OF in human body. The signal intensity and resolution can be greatly improved by utilization of a MeCN–water mixture as sample solvent. This work demonstrates that CE-ECL could be developed into a reliable tool for biofluid analysis and pharmacokinetic study for EN and OF.

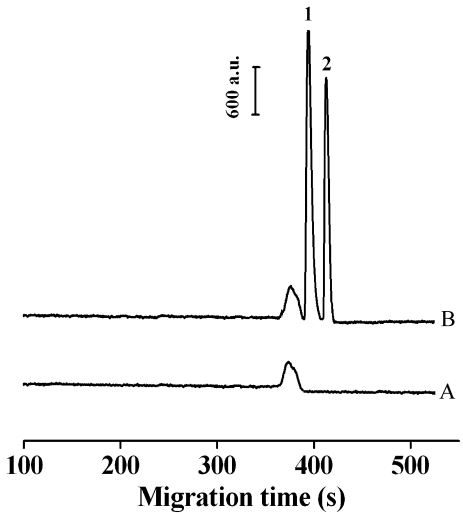

Figure 5. Electropherogram of the mixture of MeCN-H_2O (A) and EN and OF standard samples in MeCN-H_2O (B). Peak 1, 3 µmol/L EN; peak 2, 5 µmol/L OF. Conditions: electrophoretic buffer, 15 mmol/L phosphate buffer at pH 9.0; electrokinetic injection, 10 kV × 10 s; separation voltage, 15 kV; detection potential, 1.15 V; ECL solution, 5 mmol/L $Ru(bpy)_3^{2+}$ with 50 mmol/L PBS at pH 8.0.

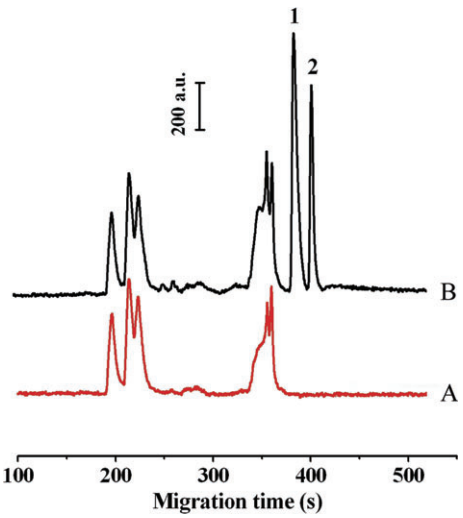

Figure 6. Electropherograms of the blank urine sample (A) and the urine sample spiked with 10 µmol/L EN and 15 µmol/L OF. (B). Peak 1, EN; peak 2, OF. Conditions are the same as in Fig. 5.

Table 1. The performance characteristics of the proposed method

Analytes	Linear range (µmol/L)	Calibration curves			LOD (mol/L)	LOQ in urine (mol/L)	LOQ in serum (mol/L)
		Slope	Intercept	*r*			
Enoxacin	0.5–50	6577	79941	0.9973	9.0×10^{-9}	3.2×10^{-7}	4.1×10^{-7}
Ofloxacin	0.5–50	16,862	154,252	0.9983	1.6×10^{-8}	5.4×10^{-7}	6.9×10^{-7}

Table 2. Recoveries of EN and OF in human urine and serum samples at different spiked level

Samples	Added (μmol/L)	Found (μmol/L)	Recovery (%)	RSD (%) ($n = 5$)
Urine		Enoxacin		
	10.0	10.4	104.0	3.8
	100.0	101.9	101.9	2.2
	300.0	292.9	97.6	1.5
		Ofloxacin		
	10.0	10.5	105.0	2.3
	100.0	95.4	95.4	4.7
	300.0	303.4	101.1	1.4
Sera		Enoxacin		
	5.0	4.8	96.0	4.6
	200.0	203.8	101.9	1.9
	400.0	399.6	99.9	2.6
		Ofloxacin		
	5.0	4.7	94.0	2.1
	200.0	210.0	105.0	3.1
	400.0	404.7	101.2	1.3

Table 3. Recoveries of EN and OF in eye drops at different spiked level

Samples	Labeled (μmol/L)	Added (μmol/L)	Total found (μmol/L)	Recovery (%)	RSD% (n = 5) Migration time	Peak area
Enoxacin						
1	5.0	0	4.6	92.0	0.8	2.1
2	5.0	15.0	20.2	101.3	0.7	1.1
3	5.0	35.0	39.2	97.7	1.2	2.7
Ofloxacin						
1	5.0	0	4.7	94.0	1.1	2.4
2	5.0	15.0	21.0	106.7	0.5	3.2
3	5.0	35.0	40.3	100.9	0.6	1.9

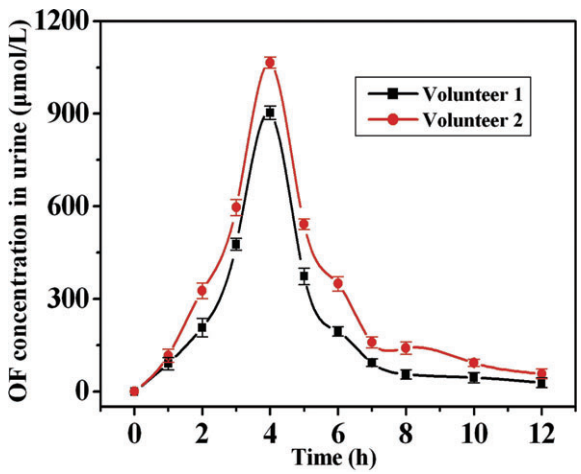

Figure 7. Urine concentration vs time profile of OF about 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 h from two healthy volunteers following a single oral dose of 300 mg.

Acknowledgements

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