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Research Article

Determination of levofloxacin and norfloxacin by capillary electrophoresis with electrochemiluminescence detection and applications in human urine

A novel method for the determination of norfloxacin (NOR) and levofloxacin (LVX) was developed by CE separation and electrochemiluminesence detection (ECL). The methods for capillary conditioning and the effect of solvent type were studied. Parameters affecting the CE and ECL were also investigated. Under the optimum conditions, the two analytes were well separated within 9 min. The LODs (S/N = 3) in standard solution are 4.8×10^{-7} mol/L for NOR and 6.4×10^{-7} mol/L for LVX, respectively. The precisions of intraday and interday are less than 4.2 and 8.1%, respectively. The LOQs (S/N = 10) in real human urine samples are 1.2×10^{-6} mol/L for NOR and 1.4×10^{-6} mol/L for LVX, respectively. The applicability of the proposed method was illustrated in the determination of NOR and LVX in human urine samples and the monitoring of pharmacokinetics for NOR. The recoveries of NOR and LVX at different levels in human urine samples were between 84.3 and 92.3%.

Keywords:
Capillary electrophoresis / Electrochemiluminescence / Levofloxacin / Norfloxacin / Pharmacokinetics / Urine analysis DOI 10.1002/elps.200800048

1 Introduction

The CE technique has become an important analytical tool because of its powerful separation efficiency, short analysis time, and minimum consumption of sample and reagents. The commonly used detection modes in CE are UV [1], LIF [2], MS [3], and electrochemical detectors (ED) [4]. In comparison with other detection modes, chemiluminescence (CL) has led to an alternative and interesting detection scheme owing to its high sensitivity and simplicity [5, 6]. Recently, CE-CL has been used for the analysis of various analytes including metal ions [7], amino acids [8], proteins [9], catecholamines [10], and antigen-antibody complexes [11].

Electrochemiluminescence (ECL) detection is a special chemiluminescence where chemiluminescence emission correlates directly or indirectly with oxidation or reduction at an electrode surface. Up to now, it has become an important and valuable detection technique and has been reviewed in literature [12–14]. The marriage of CE to ECL (CE-ECL) has proved to be a sensitive and efficient analytical technique. ECL based on tris-(2,2'-bipyridyl)-ruthenium ($Ru(bpy)_3^{2+}$) has received considerable attention since the first report [15]. CE-ECL with $Ru(bpy)_3^{2+}$ system has been successfully demonstrated in the analysis of amino acids [16, 17], proteins [18] and drugs [19–22].

Norfloxacin (NOR) and levofloxacin (LVX) are fluorinated quinolones. They exhibit high potency, a low incidence of resistance, high oral bioavailability, extensive tissue penetration, low protein binding and long elimination half-lives [23]. Owing to their favorable antibacterial and pharmacokinetic profiles, they are widely used in human and veterinary medicine. Therefore, the efficient separation and determination of NOR and LVX are very important in biological fluids, such as urine, plasma and serum. HPLC and CE have been used in the determination of NOR [24–31] or LVX [32–34]. Flow injection analysis (FIA) has been used in the simultaneous determination of NOR and LVX [35]. In CE, the detection modes used include UV [24, 32], diode array detection (DAD) [25, 26], UV photo-diode array detection [27] and LIF [28, 29]. Among these methods, little attention has been paid to the simultaneous determination of NOR and LVX and pharmacokinetic studies. A method of simple and high selectivity is thus greatly needed in analysis and pharmacokinetics studies in biological fluids such as urine.

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Abbreviations: ECL, electrochemiluminescence; **NOR**, norfloxacin; **LVX**, levofloxacin

In this work, a novel method for the simultaneous determination of NOR and LVX with CE-ECL was developed. The methods for capillary treatment and the effect of solvent type were studied. The conditions for the CE separation and ECL detection were systematically optimized. The proposed method was successfully applied in the analysis of NOR and LVX in human urine samples. The pharmacokinetics of NOR in human body was also investigated.

2 Materials and methods

2.1 Materials

Levofloxacin hydrochloride (97.3%) and NOR (98%) were purchased from National Institute for The Control of Pharmaceutical and Biological Products (Beijing, China). NOR capsules were acquired from Shanghai Yan'an pharmaceutical (Shanghai, China). Tris(2,2'-bipyridyl) ruthenium(II) chloride hexahydrate was purchased from Alfa Aesar (A Johnson Matthey Company, Ward Hill, MA). All other Chemicals were of analytical-reagent grade and used without further purification. The 10 mmol/L stock solutions of NOR and LVX were prepared by dissolving the standard samples in 0.1 mol/L HCl and stored in the dark [36]. A series of working standard solutions were prepared by diluting the stock solution with ACN (MeCN)-water mixtures with 70% v/v of MeCN. All other solutions were prepared with deionized water (18.2 MΩ·cm) processed with an Ultrapure Water System (Kangning Water Treatment Solution Provider, China). They were stored in the refrigerator at 4°C and filtered through 0.22 μm cellulose acetate filters (Shanghai Xinya Purification Material Factory) before use.

2.2 CE-ECL apparatus

The CE-ECL experiments were performed on a model MPI-A CE-ECL system ((Xi'an Remax Electronic Science-Tech, Xi'an, China). The system provided a programmable high-voltage power supply (0–20 kV), an electrochemical potentiostat, a multifunction chemiluminescence detector and a multichannel data collection analyzer.

The end-column ECL cell was composed of a 500 μm platinum (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. 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. About 300 μL of $Ru(bpy)_3^{2+}$ solution was added into the cell before analysis, and replaced every 2 h to eliminate depletion effect or potential interference from reaction products built up during the analysis.

2.3 Procedure

All separations were performed in a 55 cm long fused-silica capillary with 50 μm id and 375 μm od (Yongnian Reafine Chromatography, 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) methanol/water solution), 1.0 mol/L NaOH, 1.0 mol/L HCl, and H_2O for 15 min, and finally with electrophoretic buffer for 30 min. At the beginning of each day, the capillary was flushed with 0.1 mol/L NaOH, 0.1 mol/L HCl, water, and equilibrated with the electrophoretic buffer for 5 min successively to maintain an active and reproducible inner surface. Between each run, the capillary was rinsed sequentially with 0.1 mol/L NaOH, 0.1 mol/L HCl, H_2O, and electrophoretic buffer for 2 min. The voltage of photomultiplier tube (PMT CR105, Beijing Binsong Photonics, China) for collecting the ECL signal was set at - 800 V in the process of detection. The working electrode was biased at 1.15 V. Electrokinetic injections were performed at 10 kV for 10 s (the injected volume *ca.* 11.8 nL). 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) with 50 mmol/L PBS was added in the detection cell. The peak area was used for the analysis.

2.4 Urine sample preparation

The fresh human urine samples of two healthy male volunteers were acquired from Xinyang Central Hospital. NOR (200 mg) was orally administered to the volunteers in pharmacokinetics study. About 2 mL of urine samples were collected at 1, 2, 3, 4, 6, 8, 12 and 24 h after oral administration of 200 mg NOR, respectively. The volunteers were asked to drink sufficient and comparable amounts of water through the collection period. Blank urine was also collected just before oral administration of NOR for the preparation of spiked samples and calibration curve.

The 1.5 mL urine sample or the spiked standards were pipetted into clean 10 mL centrifugation tubes. MeCN (3.5 mL) was added to each sample and the tubes were capped. The samples were centrifuged for 10 min at 2000 rpm to remove deposit. 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 samples away from light.

3 Results and discussion

3.1 Effect of different capillary conditioning

In CE practice, the different approaches for capillary conditioning have great influence on the electrophoretic performance [37]. To obtain the reproducible results, two methods for capillary conditioning were used in the process of

experiments. Method A: at the beginning of each day, the capillary was sequentially flushed with 0.1 mol/L NaOH, water, and equilibrated with the electrophoretic buffer for 5 min. Between injections, the capillary was rinsed successively with 0.1 mol/L NaOH, H_2O, and electrophoretic buffer for 2 min. Method B: at the beginning of each day, the capillary was sequentially flushed with 0.1 mol/L NaOH, 0.1 mol/L HCl, water, and equilibrated with the electrophoretic buffer for 5 min. Between injections, the capillary was rinsed successively with 0.1 mol/L NaOH, 0.1 mol/L HCl, H_2O, and electrophoretic buffer for 2 min. The results showed that without treatment of the capillary with 0.1 mol/L HCl (method A), the migration time is shorter, but the two peaks can not be separated completely. When method B was applied, the migration time was prolonged, but good separation efficiency of two analytes could be obtained. By weighing the migration time and the separation efficiency, the method B emerged as the favorable procedure for capillary conditioning.

3.2 Effect of solvent type

NOR and LVX are poorly soluble in water, methanol *etc*. However, they have good solubility in HCl and NaOH. At first, water was used as sample solvent, but the experimental results showed that the sensitivities of NOR and LVX in water were not satisfactory. MeCN is a low-conductivity sample solvent. Shihabi [38] reported that biological samples such as urine could be stacked by MeCN. To obtain the high sensitivity and good separation efficiency, several kinds of solvents including electrophoretic buffer (20 mmol/L PBS), 0.1 mol/L HCl, 10 mmol/L NaOH, water and MeCN-water mixtures were used; the results are shown in Fig. 1. It can be seen that the sensitivity and resolution (Rs) can be improved markedly by use of MeCN-water mixture. The reason is that the addition of MeCN may improve the solubility of analytes and result in field-amplified sample stacking in CE [5]. The effects of MeCN-water mixtures with the different ratio of MeCN to water (v/v) were tested as shown in Fig. 2. The sensitivity of the analytes increased with increase of the concentration of MeCN. The increased Rs were also obtained as the concentration of the MeCN increased up to 70% v/v (Fig. 2, inlet). Further increasing the concentration of MeCN, a loss of the peak resolution was observed. By weighing both the sensitivity and the Rs, 70% v/v MeCN was selected.

3.3 Effect of detection potential

The applied potential is a key parameter for ECL detection. The intensity of the emitted light significantly correlates with the rate of the oxidation of the ECL reagent $Ru(bpy)_3^{2+}$, and this oxidation rate is related to the potential applied to the working electrode [39]. The effect of the working potential on the ECL responses in a potential range from + 1.05 to + 1.35 V was measured and the results are illustrated in Fig. 3. As shown in Fig. 3, the highest ECL intensity was

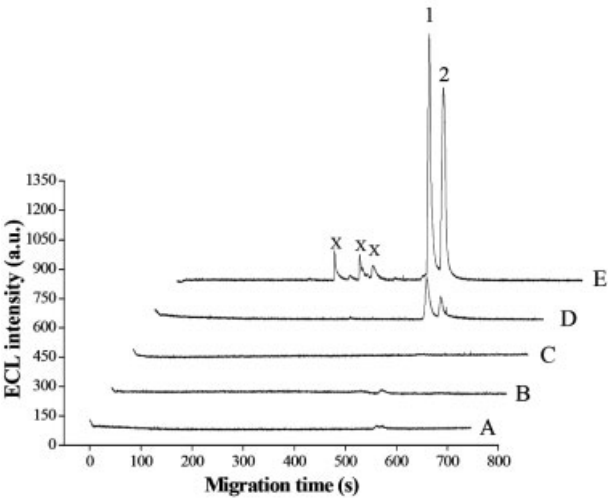

Figure 1. Electropherograms of the different sample solvents (A) electrophoretic buffer, (B) 0.1 mol/L HCl, (C) 10 mmol/L NaOH, (D) water, (E) MeCN-water mixture with 70% v/v MeCN; peak 1, 5×10^{-5} mol/L NOR; peak 2, 4.4×10^{-5} mol/L LVX, peak X, unknown compounds. Conditions: electrophoretic buffer, 20 mmol/L PBS at pH 8.0; electrokinetic injection, 10 s × 10 kV; separation voltage, 18 kV; ECL solution, 5 mmol/L $Ru(bpy)_3^{2+}$ with 50 mmol/L PBS at pH 8.20; detection potential, 1.15 V.

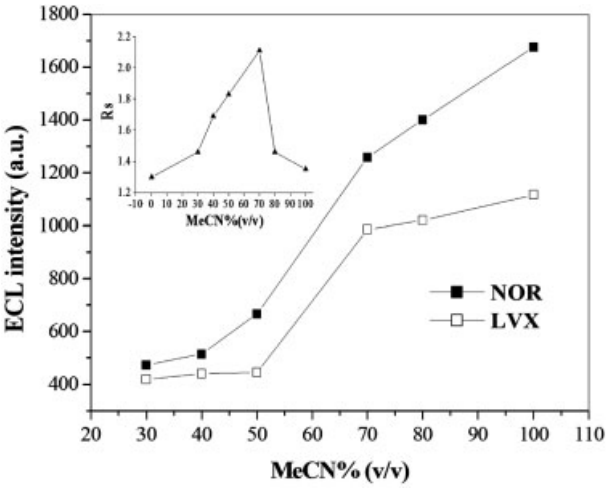

Figure 2. Effect of concentration of MeCN on ECL intensity. Conditions as in Fig. 1.

achieved at 1.15 V for both the analytes. When the potential exceeded 1.15 V, the ECL responses decreased, possibly due to the negative effect of the oxidation of water. Therefore, the optimum detection potential for NOR and LVX should be set at 1.15 V.

3.4 Effect of buffer pH in detection cell

The pH of buffer in detection cell has a significant effect on the ECL response. The buffer pH from 7.0 to 9.0 was exam-

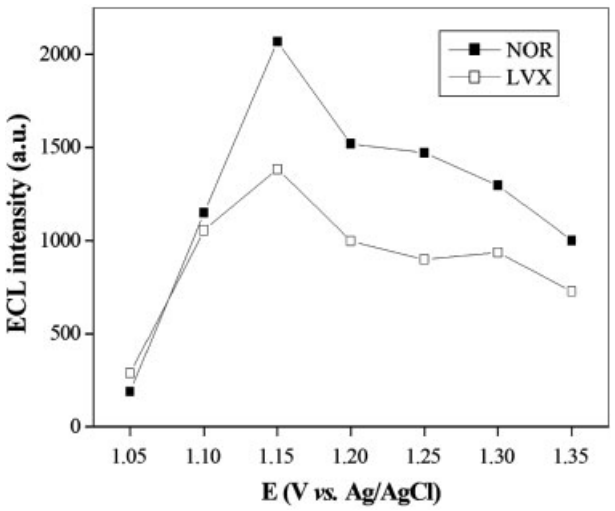

Figure 3. Effect of the detection potential on ECL intensity of 4.4 $\times 10^{-5}$ mol/L LVX and 5×10^{-5} mol/L NOR. Other conditions as in Fig. 2.

ined. The results showed that the ECL intensities of the analytes increased with the pH from 7.0 to 8.2 and then decreased. Therefore, pH 8.2 was chosen for the further experiments.

3.5 Effects of concentration and pH of electrophoretic buffer

The concentration and pH of electrophoretic buffer affect the migration time and the ECL intensity of analytes. Effects of the PBS electrophoretic buffer concentration were investigated in the range of 10 to 30 mmol/L. It can be seen in Fig. 4A that the S/N increased with the increase of the PBS concentration up to 20 mmol/L and then decreased. At the same time, the migration time became longer and the peaks became broader. Therefore, 20 mmol/L PBS was selected as the optimum electrophoretic buffer concentration.

The effect of electrophoretic buffer pH on the S/N was also investigated in the range of 7.0 to 8.5. The results showed that the S/N increased with the increase of pH from 7.0 to 8.0 (shown in Fig. 4B). In addition, the peaks of NOR and LVX overlapped partially in the range of pH 7.0 to 7.8. Both the highest S/N and the effective separation were obtained at pH 8.0. Therefore, pH 8.0 was chosen as optimum.

3.6 Effect of separation voltage

The separation voltage makes an impact on migration time and Rs. Migration time became shorter gradually with the separation voltage increase from 10 to 20 kV. However, Rs became poor (see Fig. 5A) as well as baseline noise when voltage exceeded 18 kV. Figure 5B shows that data were not consistent with Ohm's law probably due to the influence of the Joule heating effect above 18 kV. Additionally, the ECL intensity decreased when the separation voltage was higher than 18 kV. The reason is that the high separation voltage caused the strong flow of effluent from the capillary and thus resulted in the dilution of the concentration of $Ru(bpy)_3^{3+}$ in diffusion layer of working electrode surface. Therefore, 18 kV was chosen.

3.7 Performance characteristics of the method

Under the above-mentioned optimized conditions, the two analytes were well separated within 9 min. Figure 1E gives the typical electropherogram of standard solution. The three small unknown peaks appearing in Fig. 1E belong to blank solvent solution and they do not interfere with the separation of the two analytes. The calibration curves were calculated 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 0.999. LOD was considered the minimum analyte concentration yielding an S/N = 3.

The precision of the proposed method was studied by assaying three concentration levels: 5, 50 and 150 μmol/L of NOR and LVX within a day (intraday) and in 3 days (interday). The precision (measured by RSD %, $n = 5$) of NOR ranged from 1.8 to 3.4% within a day and from 4.1 to 7.1% in 3 days, respectively. The RSD ($n = 5$) of LVX ranged from 2.1 to 4.2% within a day and from 5.4 to 8.1% in 3 days, respectively.

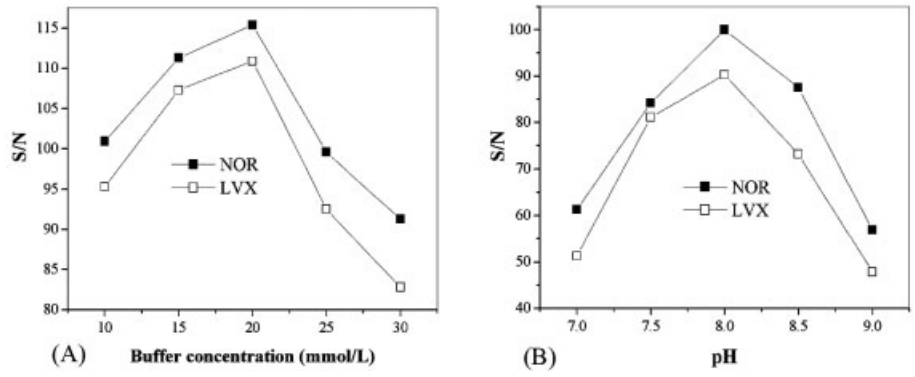

Figure 4. Optimization of the electrophoretic conditions: (A) buffer concentration and (B) buffer pH. Electrokinetic injection: 10 kV for 10 s; 4.4×10^{-5} mol/L of LVX and 5×10^{-5} mol/L of NOR. Other conditions are the same as in Fig. 2.

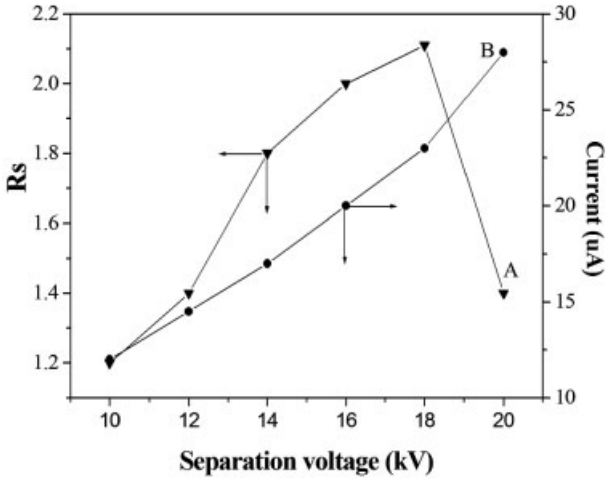

Figure 5. Effect of separation voltage on Rs (A) and electrophoretic current (B). Other conditions are the same as in Fig. 2.

Table 1. Performance characteristics of the proposed method

Substance names	Linear range (μmol/L)	Calibration curves			LOD (mol/L)
		Slope	Intercept	*r*	
LVX	2–200	650.6	9417.9	0.9991	6.4×10^{-7}
NOR	1–300	406.4	5212.6	0.9993	4.8×10^{-7}

3.8 Applications

The present method was applied to the determination of NOR and LVX in human urine samples. The typical electropherograms of blank urine sample and urine sample spiked with 5×10^{-5} mol/L NOR and 4.4×10^{-5} mol/L LVX are shown in Fig. 6. The results showed that the two analytes could be well separated in urine samples. From the urine analysis, the LOQs (defined as the lowest analyte concentration yielding an S/N = 10) were 1.2×10^{-6} mol/L for NOR and 1.4×10^{-6} mol/L for LVX, respectively. The recoveries of the two analytes at three spiked concentration levels were carried out and varied between 84.3–90.1% for NOR and 86.1–92.3% for LVX in urine samples (listed in Table 2), respectively. The RSDs of peak area were less than 7.7%.

In the pharmacokinetic study, two healthy male volunteers received an oral administration of 200 mg of NOR. The urine samples were treated and analyzed before oral administration, and at 1, 2, 3, 4, 6, 8, 12 and 24 h after oral administration. NOR is a synthetic drug and not an endogenous compound, thus it is not detected before oral administration. The results of the two volunteers are presented in the concentration-time profile of Fig. 7. As shown in Fig. 7, the content of NOR in the volunteers achieved a maximum value at 6 h after oral administration and then decreased. In addition, it can be seen that NOR does not appear to be completely metabolized, as some part of an oral dose is extracted unchanged in the urine. That is, NOR can not be completely absorbed from the gastrointestinal tract. The concentration-time profile also showed that the content of NOR in urine from volunteer 1 was higher than that from volunteer 2 at any time after oral administration. This might be caused by the actual metabolizability different from person to person.

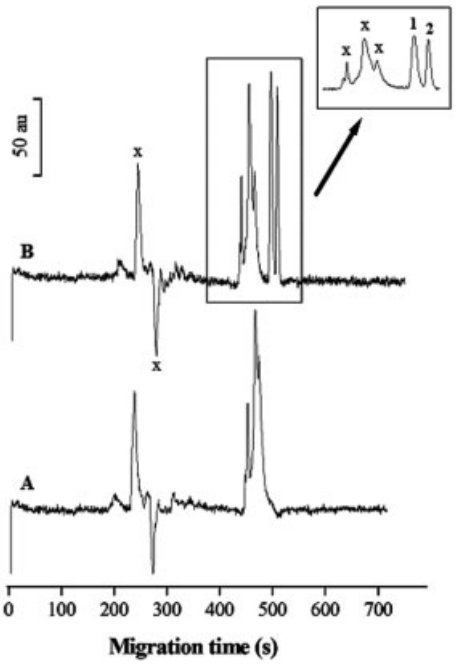

Figure 6. Electropherograms of the blank urine sample (A) and the urine sample spiked with 5×10^{-5} mol/L NOR and 4.4×10^{-5} mol/L LVX (B). Peak 1, NOR; peak 2, LVX; X, unknown compounds. Other conditions are the same as in Fig. 2.

Table 2. Recovery for two analytes at different spiked levels in human urine samples

Added (μmol/L)	Recovery (%)	RSD (%) (*n* = 5)
NOR		
80	84.3	6.3
200	90.1	7.2
400	88.3	5.7
LVX		
100	92.3	5.4
300	87.6	7.7
500	86.1	6.9

4 Concluding remarks

A novel CE-ECL method was developed for the determination of NOR and LVX. The ECL intensity and separation efficiency can be greatly enhanced by utilization of an MeCN-

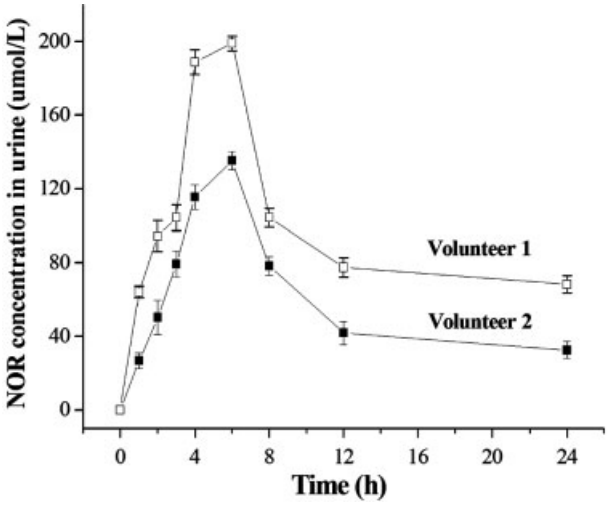

Figure 7. Urine concentration-time profile of NOR from two healthy volunteers, following a single oral dose of 200 mg, respectively.

water mixture as sample solvent. The proposed method was successfully applied to the determination of NOR and LVX in human urine and the monitoring of pharmacokinetics. This work exploits a new application of CE and demonstrates that CE-ECL could develop into a simple, novel and practical method in clinical and biochemical analysis.

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