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Direct electrogenerated chemiluminescence detection in high-performance liquid chromatography for determination of ofloxacin

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ABSTRACT

Ofloxacin (OFLX) exhibited strong electrogenerated chemiluminescence (ECL) in $NaNO_3$ solution with a dual-electrode system when constant current was exerted. Based on this observation, a sensitive direct ECL method coupled with high-performance liquid chromatography (HPLC) separation was developed for determination of OFLX in human serum. Factors affected the ECL emission were investigated. Under the optimal conditions, the ECL intensity has a linear relationship with the concentration of OFLX in the range of 1.0×10^{-8} to 4.0×10^{-6} g mL^{-1} and the detection limit was 4×10^{-9} g mL^{-1} (S/N = 3). The proposed method was sensitive, simple and convenient to operate.

1. Introduction

Ofloxacin (OFLX, (±) 9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperaziny)-7-oxo-7*H*-pyrido [1,2,3-de]-1,4-benzoxazine-6-carboxylic acid; Fig. 1) is one of the most frequently used fluorinated quinolones antibiotics in the world [1]. It has a broad spectrum of activity against gram-positive and gram-negative aerobic and facultatively anaerobic bacteria, chlamydiae, and some related organisms, such as mycoplasmas or mycobacteria [2].

Many analytical methods have been reported for the determination of OFLX, including polarographic method [3], flow injection spectrophotometric [4], chemiluminescence [5,6], capillary electrophoresis using laser-induced fluorescence detection [7], electrogenerated chemiluminescence (ECL) [8], and high-performance liquid chromatography (HPLC) with UV detection [9,10], fluorescence detection [11–13], tandem mass spectrometry detection [14], photoinduced fluorimetric detection [15] and chemiluminescence detection [16]. Although these methods have been successfully applied to analysis of OFLX in various matrices, some of these methods lack sensitivity and suffer from tedious procedure, time consuming or high cost.

ECL has become an important and valuable detection method in analytical chemistry in recent years. ECL is the process where species generated at electrodes undergo electron transfer reactions to form excited states that emit light [17]. ECL detection, emerging as a very sensitive mode of detection, has many advantages [18]. (i) The absence of an excitation light source produces a low background signal and allows a highly sensitive detection without expensive instrumentation. (ii) The electrochemical initiation of the ECL reaction and variations of the potential introduce a great temporal control over the reaction and can improve selectivity. (iii) The

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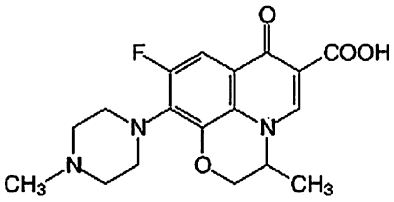

Fig. 1 – The structure of OFLX.

generation of light in the vicinity of the electrode gives improved spatial control for the sensitive detection of analytes. (iv) Wide linear range (about 3–4 orders) of responding signal benefits the quantitative detection of analytes. The majority of analytical applications of ECL now appearing in the literature are concerned with the indirect ECL of $Ru(bpy)_3^{2+}$ [19,20] and luminol [21,22]. In contrast with indirect ECL, organic molecules having structural character capable of direct ECL have been explored relatively infrequently when compared to $Ru(bpy)_3^{2+}$ and luminol ECL. To our best knowledge, only a few researches of direct ECL have been done for analytical use in open literature [23–25] and none of these studies have been exploited for post-column detection of high-performance liquid chromatography.

In our present work, strong ECL emission was observed when a suitable direct current was applied to the working electrode (Pt) immersed in a solution of OFLX and $NaNO_3$. Based on this observation, a sensitive direct ECL method coupled with HPLC separation was developed for determination of OFLX. Factors affected the ECL emission of OFLX were investigated. Under the optimal conditions the method has been used for determination of OFLX in human serum.

2. Experimental

2.1. Apparatus and reagents

The experimental setup for HPLC–ECL is shown in Fig. 2(a). The analyses were carried out using a LC-6A (Shimadzu, Tokyo, Japan) liquid chromatography equipped with a Rheodyne 7725i syringe loading sample injector valve (Cotati, CA, USA) and a Nucleosil C_{18} column (I.D.: 250 mm × 4.6 mm, particle size: 5 μm, pore size: 100 Å, Macherey-Nagel, Düren, Germany). A dual-electrode electrochemical flow-cell (Fig. 2(b)) was used for the ECL experiments. The constant current was supplied by a JH2C galvanostat (Shanghai Electric Instrument Plant, Shanghai, China). The polymethylmethacrylate-resin-housed flow-cell contained a coiled Pt wire working electrode (Ø 0.2 mm) and a stainless steel counter electrode attached to the flow-cell outlet and positioned close to and directly opposite the Pt working electrode. The flow-cell was placed close to the window of the photomultiplier tube. The emission from the counter electrode was prevented by masking flow-cell with a black tape placed on the outside of the flow-cell and opposite the counter electrode. Data collection and processing were performed using an IFFL-D Flow Injection Chemiluminescence Analyzer (Xi'an Remax Electronic Science-Tech Co. Ltd., Xi'an, China).

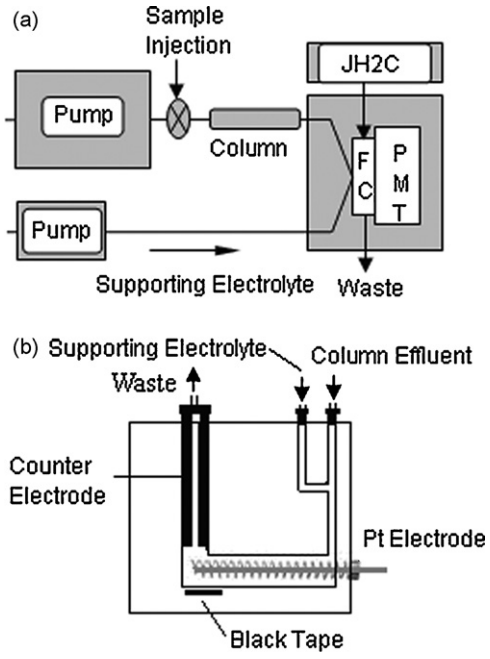

Fig. 2 – Schematic diagram of HPLC–ECL system (a) and side view of FC (b). FC, flow-cell; PMT, photomultiplier tube; JH2C, galvanostat.

OFLX was obtained from the Institute of Pharmaceutical and Biomaterial Authentication of China (Beijing, China). Acetonitrile (ACN) was of HPLC grade (Tianjin Kermel Chemical Reagent Co., Ltd., Tianjin, China). All the other reagents were of analytical reagent grade unless specified otherwise. Deionized and doubly distilled water was used throughout.

2.2. Preparation of serum samples

To 0.5 mL each of the spiked test samples (5.0, 10.0, 20.0, 40.0, 60.0 ng mL^{-1}), 0.3 mL of 7% perchloric acid was added, vortex-mixed for 1 min and centrifuged at 2500 rpm for 15 min. A 20 μL volume of the supernatant was directly injected to the column [13].

2.3. Procedure

The mobile phase was pumped through the column at a flow rate of 1.0 mL min^{-1}. The column effluent was mixed with the supporting electrolyte (2.5 mL min^{-1}) at a mixing-tee and then flowed into the flow-cell. Until stable baseline was achieved, 20 μL standard solution or sample solution was injected into the mobile phase. The quantitative determination was based on the net ECL intensity $I = I_S - I_0$, where I_S is the ECL intensity in the presence of OFLX and I_0 is the intensity of blank signal.

3. Results and discussion

3.1. Optimization of the ECL system

Our preliminary investigations showed that OFLX gave ECL emission at Pt working electrode when KNO_3 was used as the

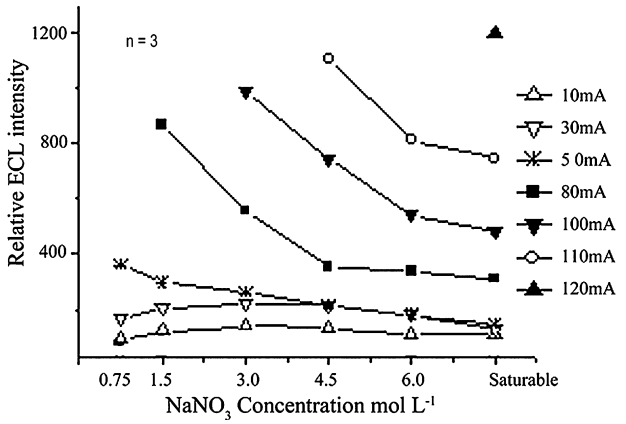

Fig. 3 – Effects of $NaNO_3$ concentration and current density on the ECL intensity of OFLX. *n*, number of determination; mA: milliampere.

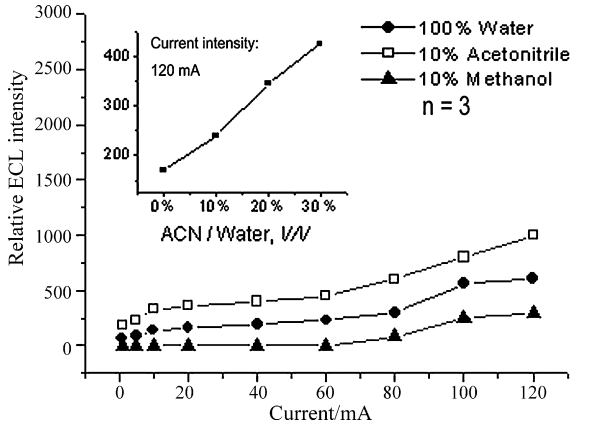

Fig. 4 – ECL emission of OFLX in varied medium. *n*, number of determination; mA: milliampere.

supporting electrolyte. The previous studies have shown that the piperazine ring was susceptible to oxidation [26–29]. A similar mechanism is believed to be operative in the oxidation of OFLX in present work. Therefore, the possible CL mechanism of this reaction may be attributed to the following reactions in its simplest form.

$$\text{OFLX} \xrightarrow{-e} [\text{O}]^*_{\text{OFLX}} \rightarrow [\text{O}]_{\text{OFLX}} + h\nu$$

where $[O]_{OFLX}$ is the oxidation product of OFLX and $[O]^*_{OFLX}$ is the excited state of $[O]_{OFLX}$.

Further experiments compared the ECL intensity of OFLX in different supporting electrolyte (1.0 mol L^{-1}) including KCI, $NaNO_3$, KNO_3, $KClO_4$ and Na_2SO_4. The results showed that the ECL intensity in KCI or Na_2SO_4 solution was very weak, but strong in KNO_3 or $NaNO_3$. In $KClO_4$, the intensity is approximately 50% of the intensity obtained in KNO_3 or $NaNO_3$. In our present work, $NaNO_3$ was selected as supporting electrolyte because of its high solubility in water.

The effects of $NaNO_3$ concentration and the current intensity on the ECL intensity were also optimized. As shown in Fig. 3, the ECL intensity increased with the increase of current intensity at each level of the $NaNO_3$ concentration, but at each level of the current intensity, the effect of $NaNO_3$ concentration on the ECL intensity was varied. When the current intensity was lower than 30 mA, the maximal relative ECL intensity could be reached at 3.0 mol L^{-1} when increased the $NaNO_3$ concentration from 0.75 mol L^{-1} to a saturated solution, while the ECL intensity decreased with the increase of $NaNO_3$ concentration when the current was higher than 30 mA. To obtain the maximal relative ECL intensity, a 120 mA current and a saturated $NaNO_3$ solution were selected for subsequent studies. It should be noted that, although a large quantity of bubbles were generated when higher current intensity was applied, satisfactory results of precision and sensitivity were obtained as shown in later section.

The acidity and alkalinity of the supporting electrolyte were investigated by adding varied concentrations of HNO_3 and NaOH into the supporting electrolyte. The results showed that the ECL intensity decreased with the increase of the HNO_3 concentration from 1.0×10^{-3} to 0.1 mol L^{-1} or NaOH concentration from 1.0×10^{-5} to 0.1 mol L^{-1}, and remained nearly stable in a broad interval from 1.0×10^{-3} mol L^{-1} HNO_3 to 1.0×10^{-5} mol L^{-1} NaOH. It indicated that the ECL of OFLX could be performed in an unbuffered system.

For separation of OFLX, a mixture of acetonitrile and water or a mixture of water, acetonitrile and methanol was generally used. The ECL emission of OFLX in the medium of water, acetonitrile/water (10%, v/v) and methanol/water (10%, v/v) were compared. As shown in Fig. 4, the ECL intensity in 10% acetonitrile was higher than that in 100% water and in 10% methanol, while in the medium of acetonitrile/water, the ECL intensity increased with the increase of the ratio of acetonitrile/water from 0 to 30%.

3.2. *Method performance*

Under the optimum conditions as those described above, the calibration graph of ECL intensity versus OFLX concentration was linear in the range of 1.0×10^{-8} to 4.0×10^{-6} g mL^{-1}; The regression equation was $I = 803.7C + 81.4$ (C: OFLX concentration, 10^{-6} g mL^{-1}) with a correlation coefficient of 0.9963. The detection limit was 4×10^{-9} g mL^{-1} (S/N = 3), which was lower than that of HPLC with UV detection (0.2 ng μL^{-1}) [30], fluorescence detection (20 ng mL^{-1}) [31] and tandem mass spectrometry detection (21 ng mL^{-1}) [14].

3.3. *Sample analysis*

For separation and detection of OFLX in serum sample, the mobile phase was a mixture of ACN–water–H_3PO_4–triethylamine (30:70:0.1:0.1, v/v/v/v, pH 3.5). HPLC separation of OFLX was performed isocratically at a flow-rate of 1.0 mL min^{-1} and the eluate from the column was continuously monitored by ECL. Fig. 5 shows typical chromatograms of OFLX-free human serum sample (a), standard OFLX solution (b) and human serum sample spiked with OFLX. It can be seen from the chromatograms that OFLX was separated as a single peak without other interference.

Following the procedure described in Section 2.3, the proposed method was applied to the analysis of OFLX in human serum. The validation of the proposed method and a recovery

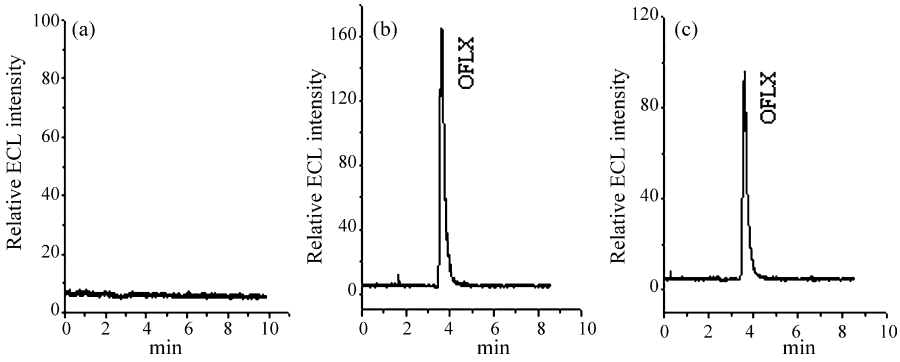

Fig. 5 – Typical chromatograms of (a) a blank serum sample, (b) a standard solution of OFLX (100 ng mL^{-1}) and (c) a serum sample spiked with OFLX (50 ng mL^{-1}). Mobile phase, ACN–water–H_3PO_4–triethylamine (30:70:0.1:0.1, v/v/v/v, pH 3.5).

Table 1 – Determination of OFLX in human serum

Sample	Detected (ng mL^{-1})	Added (ng mL^{-1})	Found (ng mL^{-1}) (recovery, %)[a]	Precision (R.S.D.)	
				Intra-day (%)[b]	Inter-day (%)[c]
1	19.6	5.0	24.8(104)	4.5	6.1
		10.0	29.7(101)	3.8	5.8
		20.0	39.3(98)	4.0	6.3
2	38.5	20.0	57.8(97)	3.6	5.0
		40.0	76.8(96)	3.2	5.5
		60.0	96.2(96)	3.8	4.9

R.S.D.: relative standard deviation.

[a] Mean of three determinations.

[b] Nine repeated injections of each sample solution.

[c] Three repeated injections of each sample solution on three consecutive days.

test were carried out on spiked samples of human serum. The intra-day precision was tested with 9 repeated injections of each sample solution spiked with OFLX. The inter-day precision of the proposed method was studied by analyzing the same spiked samples, injected three times every day for each sample solution, on three consecutive days. The results were shown in Table 1.

4. Conclusions

In this paper, a new and simple direct ECL method coupled with HPLC separation was developed. The ECL reaction is well compatible with the mobile phase of HPLC. The proposed method allows for the sensitive determination of OFLX in human serum samples, and offers wider linear range, lower detection limit, and shorter analysis time. Moreover, the strong ECL emission of OFLX shows that the OFLX based molecules could be usable as ECL labels.

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