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Determination of norfloxacin using a terbium-sensitized electrogenerated chemiluminescence method

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ABSTRACT: A simple electrogenerated chemiluminescence (ECL) analysis method for the determination of norfloxacin (NFLX) is reported. It is based on ECL produced by Na_2SO_3, which is sensitized by the Tb–NFLX complex. The relative ECL intensity of the Tb^{3+}–NFLX–Na_2SO_3 system is proportional to the amount of NFLX. The optimized experimental conditions were investigated. The linear range and detection limit for NFLX were 1.0×10^{-10}–8.0×10^{-7} mol/L and 2.8×10^{-11} mol/L, respectively. This method was successfully applied to the determination of NFLX in a capsule. NFLX in urine can be directly detected without pretreatment or separation. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: electrogenerated chemiluminescence; cyclic voltammetry; terbium; norfloxacin (NFLX); sodium sulphite

INTRODUCTION

Quinolones are an important class of synthetic antibacterial agents, and the fluoroquinolones (fluorinated quinolones) show higher antibacterial activity (1). Norfloxacin (NFLX) [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline-carboxylic acid], a synthetic fluoroquinolone antibacterial agent, has been widely applied in clinical medicine. It has good oral absorption and broad–spectrum activity against many pathogenic Gram-negative and Gram-positive bacteria (2, 3). The bactericidal action of NFLX results from interference with the enzyme DNA gyrase, which is needed for the synthesis of bacterial DNA (4, 5). In chemical research, NFLX has also received much attention, e.g. it is used in the determination of some substances (6–8). Norfloxacin was used as internal standard by Barron *et al.* (6) for simultaneous determination of oxolinic acid and flumequine in spiked chicken tissue using capillary electrophoresis. Ye *et al.* (7) made the determination of yeast DNA based on its quenching the fluorescence emission of norfloxacin. In addition, reverse-flow-injection spectrophotometric determination of iron(III) was employed by Pojanagaroon *et al.* (8), using the reaction between Fe(III) with norfloxacin in ammonium sulphate solution.

Methods for NFLX determination include microbiological methods (9, 10), polarography (11), spectrophotometry (12, 13), liquid chromatography with UV-visible detection (14) or fluorescence detection (15, 16), liquid chromatography–tandem mass spectrometry (LC–MS) (17, 18), spectrofluorimetry (19, 20) and flow-injection chemiluminescence (CL) (21). Microbiological methods are slow and suffer from poor precision and specificity. Polarography is instrumentally simple but it does not provide a low detection limit. Spectrophotometry is widely used in the determination of different substances and it has good applicability, does not usually provide a very low detection limit, and interference is usually serious if it is applied to biological samples. Liquid chromatography coupled with various detection techniques (14–18) offers considerable potential as a method for the analysis of fluoroquinolones. Vílchez *et al.* (19) determined NFLX fixed on Sephadex SP C-25 gel, using solid-phase spectrofluorimetry, and obtained high sensitivity (the detection limit was 0.04 ng/mL). Three-way fluorescence data and multivariate calibration based on parallel factor analysis were combined for the simultaneous quantitation of three fluoroquinolones (norfloxacin, enoxacin and ofloxacin) by Muñoz de la Peña *et al.* (20), and a low detection limit (0.2 μg/L) for NFLX was obtained.

Chemiluminescence (CL) has been widely used in chemical and biological fields (22–27), which rely on the effects related to the chemical reaction only, i.e. without the need for an external energy supply. It is characterized by high sensitivity, a large dynamic range of concentrations of the substances determined, minimum background, no disturbances and light scattering, reproducibility, and the possibility of simple and quick analysis (28). Determination of NFLX by a CL method using a flow-injection system was studied by Liang *et al.* (21). The effect of NFLX on weak

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CL from peroxynitrous acid was applied to the determination of NFLX. The linear range and detection limit were 1.0×10^{-7}–1.0×10^{-5} mol/L and 5.9×10^{-8} mol/L, respectively.

In recent years, electrogenerated chemiluminescence (ECL) has emerged as a useful analytical technique by which a CL reaction is produced in the vicinity of an electrode surface when suitable potential is applied to it. The reagents needed for the reaction can often be electrochemically produced *in situ* and allowed to mix as soon as they form; therefore, the reaction can be controlled and manipulated by alterations of the applied potential. This technique not only retains the merits of CL analysis, but offers some important advantages over most conventional CL, such as a smaller sample consumption and better selectivity, which are inherent in both amperometric analysis and constant potential electrolysis (29). The emission is also focused on the electrode surface, which can be shaped and accurately positioned in relation to the optical measurement system for maximum sensitivity. ECL has been widely applied in different analytical science fields (30–34).

The ECL of Na_2SO_3 is very weak. The strong characteristic luminescence of Tb^{3+} emits as the complex of Tb^{3+} with NFLX is added to Na_2SO_3. The ECL intensity of the Tb^{3+}–NFLX–Na_2SO_3 system is proportional to the amount of NFLX. In the present study, the determination of NFLX in a capsule and urine samples has been studied with ECL analysis. This method provides a lower detection limit and a broader linear range than those of other methods (polarography, spectrophotometry, HPLC, etc.), including the chemiluminescence method. However, its reproducibility is not as good as the flow-injection chemiluminescence method (21). The mechanism of ECL in this system has been proposed. To the best of our knowledge, this method has not been reported previously.

MATERIALS AND METHODS

Apparatus

The ECL analyser system used for the determination of NFLX is shown in Fig. 1. Electrochemistry and ECL experiments were performed using a CHI 620 electrochemical workstation (CH Instruments). In these experiments a platinum disk electrode (0.2 cm diameter) was employed as the working electrode, with a Pt wire as the counter-electrode and a Ag/AgCl gel electrode (0.20 V vs. NHE) as the reference electrode. ECL emission intensities were measured with a BPCL ultra-weak chemiluminescence analyser (Institute of Biophysics, Academia Sinica, China). The fluorescence spectrum and the ECL spectrum were recorded with a RF-5301PC spectrofluorometer (Shimadzu, Japan).

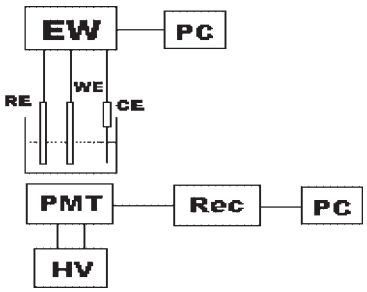

Figure 1. Schematic diagram of electrogenerated chemiluminescence analyser. WE, working electrode; RE, reference electrode; CE, counter-electrode; EW, electrochemical workstation; PMT, photomultiplier tube; HV, high voltage; Rec, recorder; PC, computer.

Reagents

All chemicals used were of analytical reagent grade and distilled, deionized water was used throughout. NFLX stock standard solution (1.0×10^{-3} mol/L) was prepared by dissolving 79.8 mg NFLX (Institute of Medicinal Biotechnology, Beijing, China) in 2 mL HCl (0.2 mol/L) and diluting with water to 250 mL. A stock solution of $TbCl_3$ (0.010 mol/L) was prepared by dissolving 186.9 mg Tb_4O_7 (General Research Institute for Nonferrous Metals, China) in 10 mL HCl (12 mol/L) at 85–95°C and evaporating the solution to near-dryness before diluting to 100 mL with water; this stock solution was diluted to the desired concentration when used. Na_2SO_3 working solution (6.0×10^{-4} mol/L) was prepared daily.

Methods

All experiments were carried out in aqueous solution containing 0.10 mol/L Na_2SO_4. Solutions used to obtain the ECL emission intensity and ECL spectrum were composed of Tb^{3+}, NFLX, Na_2SO_3 and 0.10 mol/L Na_2SO_4. ECL was obtained by sweeping from 0 to +1.2 V at 0.08 V/s via cyclic voltammetry (CV), and the maximum ECL intensity (at +0.80 V) was used in the quantitation of NFLX. The relative ECL intensity, ΔI (the difference between the ECL intensity of NFLX solution and that of the blank solution without NFLX), is proportional to the concentration of NFLX. Electrodes were cleaned after each experiment by cycling six times between –2.0 and +2.0 V vs. Ag/AgCl at 0.1 V/s in concentrated sulphuric acid solution, followed by sonication for about 20 s in dilute nitric acid (35).

Sample preparation

Ten NFLX capsules (Zhejiang Medicine Corporation) were ground into homogenized powder. Then 200 mg powder, corresponding to one capsule, was dissolved with 4 mL HCl (0.2 mol/L) in a small beaker. The solution was filtered and the residue was washed with water

five times, and then diluted with water to 100 mL. Working solution was prepared by appropriate dilution of this sample solution, so that the final concentration was within the linear range. No further pretreatment except proper dilution was required for urine samples.

RESULTS AND DISCUSSION

Optimization of experimental variables

Some experimental variables were examined to establish the optimum conditions. The optimized parameters include electrochemical parameters and the concentrations of reagents.

The effect of CV scan rate on relative ECL intensity was studied over the range 0.01–0.50 V/s. The largest ECL intensity was given by using a 0.08 V/s scan rate.

The pH of the ECL system has a very great influence on the ECL intensity. H_2SO_4 or NaOH was added to the solution to test the effect. In general, ECL intensity remains constant within the pH range 4.0–8.0 and the maximum ECL intensity was obtained at pH ≈ 7. The intensity increases with pH increasing when pH < 4.0 because the increase of $-COO^-$ in NFLX enhances the formation of the Tb^{3+}–NFLX complex, which further facilitates the dissociation of the carboxylic proton. The complex is theoretically more favourable at pH > 8.50 but the intensity decreases owing to the precipitation of terbium hydroxide (36). Therefore, no H_2SO_4 or NaOH were added in further work.

The influence of Na_2SO_3 concentration in the range 2.0×10^{-4}–1.2×10^{-3} mol/L on the ECL signal was studied. The maximum ECL intensity was observed when using 6.0×10^{-4} mol/L Na_2SO_3. Hence, this concentration was selected for the subsequent work.

The effect of Tb^{3+} concentration on ECL emission was also examined over the range 4.0×10^{-5}–1.6×10^{-3} mol/L. The results are shown in Fig. 2. Below 4.0×10^{-4} mol/L the emission intensity increased. With the concentration of Tb^{3+} increasing from 4.0×10^{-4} mol/L, constant ECL was observed. Therefore, 4.0×10^{-4} mol/L Tb^{3+} was selected for the present work.

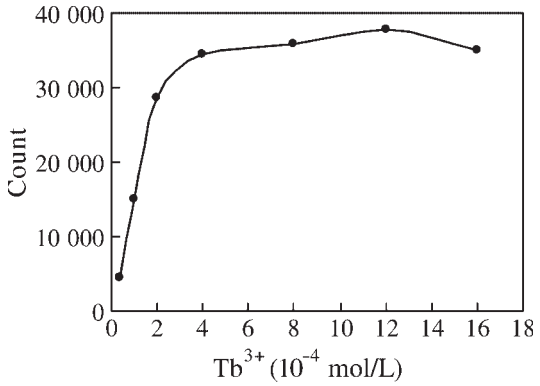

Figure 2. The effect of Tb^{3+} concentration on relative ECL intensity. C(NFLX) = 4.0×10^{-8} mol/L; C(Na_2SO_3) = 6.0×10^{-4} mol/L.

Interference studies

In order to assess the possibility of analytical application of the method, the effects of some common excipients, ions and organic compounds were investigated. The tolerable concentration ratios for interference at the 5% level were over 1000 for K^+, Ca^{2+}, Mg^{2+}, Na^+ and NH_4^+, 500 for dextrine, starch, glucose and vitamin B_1, 100 for Zn^{2+}, Mn^{2+}, Al^{3+}, Pb^{2+}, Fe^{3+}, Ni^{2+} and myoglobin, 50 for haemoglobin, and 10 for Cu^{2+} and Co^{2+} in the determination of 4.0×10^{-8} mol/L NFLX.

Analytical characteristics

The calibration graphs for the determination of NFLX were conducted under the optimal conditions, and the results are given in Table 1. The detection limit for NFLX calculated from the standard deviation of the blank (the reagent blank without NFLX, $n = 19$) (3σ) (37) is 2.8×10^{-11} mol/L and the relative standard deviation (RSD) is 4.1% for 11 repeated determinations of 4.0×10^{-8} mol/L NFLX.

Analytical applications

The proposed method was applied to the determination of norfloxacin in capsules and compared with fluorimetry (38). The calibration curve method (39) was used in the determination of norfloxacin content, and the recovery was examined by the standard addition method (40). The data are summarized in Table 2. As can be seen, there is no significant difference between the labelled content and that obtained by the proposed method and this method is obvously as accurate as fluorimetry.

Table 1. The data of calibration curves

Linear range (mol/L)	Regression equation	r	Number of data points
1.0×10^{-10}–1.0×10^{-9}	$I = 3.6192 \times 10^{12}$ C(NFLX) + 3519.5	0.9978	6
1.0×10^{-9}–1.0×10^{-8}	$I = 1.5123 \times 10^{12}$ C(NFLX) + 8859.1	0.9995	7
1.0×10^{-8}–1.0×10^{-7}	$I = 4.385 \times 10^{11}$ C(NFLX) + 7176.7	0.9992	7
1.0×10^{-7}–8.0×10^{-7}	$I = 2.3566 \times 10^{11}$ C(NFLX) + 66238	0.9988	6

Table 2. NFLX content in capsule and recovery ($n = 5$)

NFLX labelled (mg)	Amount found ± % RSD (mg)		NFLX added (nmol/L)	NFLX found (nmol/L)	Recovery ± RSD (%)
	Proposed method	Fluorimetry			
100.0[a]	103.2 ± 4.2	93.5 ± 3.2	10.0	10.5	105.3 ± 4.0
			30.0	29.3	97.6 ± 2.8
			50.0	53.1	106.3 ± 3.5
			70.0	69.1	98.8 ± 2.9
			90.0	84.9	94.4 ± 3.5

[a] The content in 200.0 mg NFLX capsule powder.

Table 3. Recovery of NFLX in urine ($n = 5$)

NFLX added (nmol/L)	NFLX found (nmol/L)	Recovery ± RSD (%)
10.0	9.4	93.9 ± 4.2
30.0	29.9	99.5 ± 2.9
50.0	50.1	100.2 ± 3.3
70.0	71.7	102.5 ± 2.5
90.0	87.5	97.2 ± 3.6

It is reported (41) that NFLX is hardly metabolized in the human body, and the concentration of NFLX in the urine of a person who took 200 mg NFLX 12 h ago was 124 μg/mL. Therefore, in order to make the sample concentrations of the drug within the linear range, urine sample was diluted 1000-fold and analysed by the standard addition method (40). The results are given in Table 3.

Possible mechanism of the Tb^{3+}–NFLX–Na_2SO_3 ECL system

The Tb^{3+}–NFLX–Na_2SO_3 system displays a chemically irreversible oxidation process with a peak potential of 0.80 V vs. Ag/AgCl in aqueous solution. Under the same conditions, independent Na_2SO_3 exhibits a same irreversible oxidation process, with a peak potential of 0.80 V; however, oxidation processes do not appear for independent Tb^{3+} or NFLX. These factors indicate that Na_2SO_3 is oxidized in the Tb^{3+}–NFLX–Na_2SO_3 system.

A representative example of ECL for the Tb^{3+}–NFLX–Na_2SO_3 system is shown in Fig. 3. Emission occurs at a potential corresponding to the oxidation of Na_2SO_3. It has been proposed that the chemiluminescence of some oxidant–sulphite systems arises from excited sulphur dioxide (SO_2*; one of the products in the oxidation of Na_2SO_3) (42–45). It is therefore postulated that SO_2* is electrochemically produced through the oxidation of Na_2SO_3 in the Tb^{3+}–NFLX–Na_2SO_3 ECL system. However, the ECL intensity of independent Na_2SO_3 is very weak because of the low luminescence efficiency of SO_2*. By introducing a fluorophore, whose absorption falls in the emission range of SO_2*,

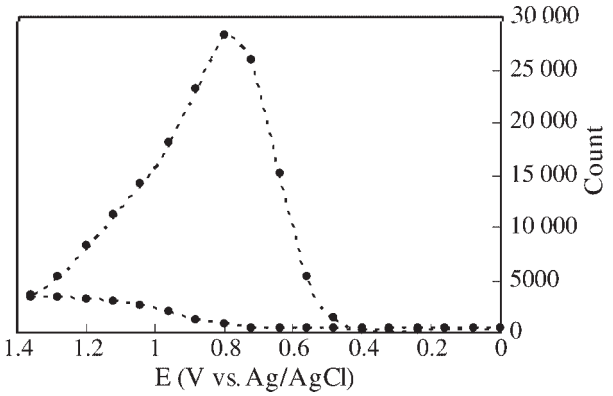

Figure 3. ECL intensity vs. potential for the Tb^{3+}–NFLX–Na_2SO_3 system. Electrode was scanned from 0.0 to 1.4 V and back to 0.0 V at a scan rate of 0.08 V/s. Tb^{3+}, 4.0×10^{-4} mol/L; NFLX, 4.0×10^{-8} mol/L; Na_2SO_3, 6.0×10^{-4} mol/L; Na_2SO_4, 0.1 mol/L.

the chemiluminescence intensity is usually enhanced through intermolecular energy transfer from SO_2* to the fluorophore (46). Based on this, Tb^{3+} or NFLX was added to the Na_2SO_3 ECL system, but no notable increase in the ECL intensity could be observed. However, when both Tb^{3+} and NFLX were added to the ECL system, the ECL intensity was greatly enhanced. The ECL spectrum of the Tb^{3+}–NFLX–Na_2SO_3 system is shown in Fig. 4. The emission peaks were located at 490, 545, 585 and 620 nm, which are the characteristic emissions of terbium ion and correspond to the transitions $^5D_4 \rightarrow {}^7F_6$, $^5D_4 \rightarrow {}^7F_5$, $^5D_4 \rightarrow {}^7F_4$ and $^5D_4 \rightarrow {}^7F_3$ (47), indicating clearly that the excited Tb^{3+} is the emitter and that there must be energy transfer in this ECL system. According to Diamandis (48), Tb^{3+} emits very weak metal ion fluorescence when excited by radiation, which is not analytically useful. The fluorescence is dramatically enhanced when Tb^{3+} forms chelates with appropriate organic ligands, which include fluoroquinolones (49). Therefore, a possible mechanism for the Tb^{3+}–NFLX–Na_2SO_3 ECL system can be explained as follows. The excitation of Tb^{3+} takes place through the intermolecular energy transfer from SO_2* produced electrochemically to the ligand, and then an intramolecular energy transfer to Tb^{3+}, followed by $^5D_4$

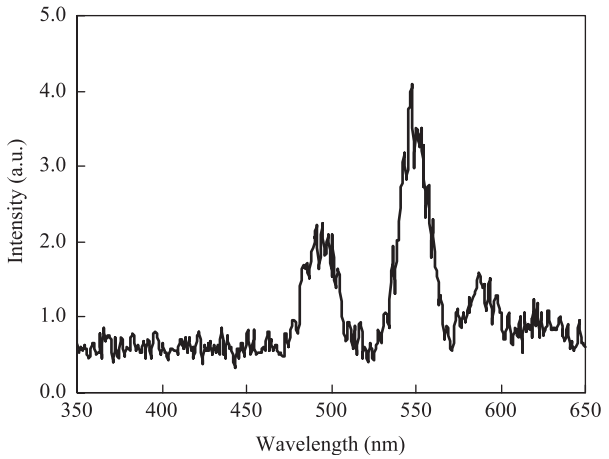

Figure 4. ECL emission spectra for the Tb^{3+}–NFLX–Na_2SO_3 system. The spectrofluorometer scanned at the fastest rate when the electrode was scanned to 0.8 V. Tb^{3+}, 4.0×10^{-4} mol/L; NFLX, 5.0×10^{-5} mol/L; Na_2SO_3, 6.0×10^{-4} mol/L; Na_2SO_4, 0.1 mol/L.

emission of Tb^{3+} in the Tb–NFLX complex. However, the process of direct energy transfer from SO_2* to $^5D_4$ of Tb^{3+} in the Tb–NFLX complex cannot be excluded, although this process is not efficient. The mechanism can be expressed as follows:

$$SO_2^* + \text{Tb–NFLX} \rightarrow SO_2 + \text{Tb–NFLX}^*$$
$$\text{Tb–NFLX}^* \rightarrow \text{Tb}^*\text{–NFLX}$$
$$\text{Tb}^*\text{–NFLX} \rightarrow \text{Tb–NFLX} + h\nu$$

CONCLUSION

From the experimental point of view, the manipulation proposed in this work is very simple and the apparatus is cheap. The ultra-weak chemiluminescence analyser combined with the electrochemical system provides sensitive and accurate results for the determination of NFLX. Therefore, the proposed ECL method represents a simple, inexpensive, high-sensitivity and -selectivity method for the determination of NFLX. This method is based on luminescence produced electrochemically by the oxidation of Na_2SO_3 and sensitized by the Tb–NFLX complex.

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