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Flow-injection chemiluminescence determination of ofloxacin using the $Ru(bpy)_2(CIP)^{2+}$–Ce(IV) system and its application

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ABSTRACT: This paper reports a flow-injection chemiluminescence method for the determination of ofloxacin (OFLX) using the $Ru(bpy)_2(CIP)^{2+}$–Ce(IV) system. Under the optimum conditions, the relative CL intensity was proportional to the concentration of OFLX in the range 3.0×10^{-8}–1.0×10^{-5} mol/L and the detection limit was 4.2×10^{-9} mol/L. The proposed method has been successfully applied to the determination of ofloxacin in pharmaceuticals and human urine. The chemiluminescence mechanism of the system is also discussed. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: ofloxacin; chemiluminescence; flow-injection; Ce(IV); $[Ru(bpy)_2(CIP)]Cl_2$

Introduction

Ofloxacin (OFLX), the third-generation member of the quinolone antibiotics, is active against both Gram-positive and Gram-negative bacteria through inhibition of their DNA gyrase and has been prevalently applied in the clinical treatment of certain infections, such as prostate, skin and urinary tract infections, because of its broad-spectrum activity against micro-organisms, good absorption and low frequency of adverse effect (1). Analytical methods, including high-performance liquid chromatography (HPLC) (2–8), fluorimetry (9–13), spectrophotometry (14), capillary electrophoresis (CE) (15–18), NMR spectroscopy (19), polarography and voltammetry (20–23) and chemiluminescence (24–28) have been reported for the determination of OFLX in pharmaceuticals, biological fluids and other samples. Zhao (27) used lanthanide ions as luminescence sensitizer to study flow-injection chemiluminescence (FI–CL) for the determination of OFLX. Wang *et al.* (29) determined OFLX using the reaction of luminol and hydrogen peroxide catalysed by gold nanoparticles, following the FI–CL method. In recent years, FI–CL has become an attractive method in analytical chemistry thanks to its high sensitivity, wide linear range, good reproductivity and relatively simple manipulation.

Bivalent ruthenium complexes, especially $Ru(bipy)_3^{2+}$ and $Ru(phen)_3^{2+}$, are sensitive CL reagents and have been used to determine many chemicals, such as organic acids (30), amino acids (31, 32), medications (33–36), sulphite (37) and some others (38–41). But the chemiluminescence (CL) of other Ru(II) complexes has rarely been reported.

A newly synthesized Ru(II) complex, $[Ru(bpy)_2(CIP)]Cl_2$ (Fig. 1; CIP: 4-carboxyl-imidado[4,5-*f*][1,10]-phenanthroline), which was synthesized according to a modified method reported previously (42), can react with Ce(IV) to give rise to CL. The introduction of OFLX can enhance the CL intensity, and the CL reaction takes place rapidly. On this basis, a FI–CL method for the determination of ofloxacin using the $Ru(bpy)_2(CIP)^{2+}$–Ce(IV) system has been developed. Its mechanism is also discussed.

Materials and methods

Reagents

All reagents used were of analytical grade. Double-distilled water was used throughout the experiments. OFLX (Institute of Medicinal Biotechnology of China, Beijing) standard stock solution (1.0×10^{-3} mol/L) was prepared by dissolving 18.1 mg OFLX in 0.5 mL HCl solution (0.2 mol/L) and diluting with water to 50 mL. $[Ru(bpy)_2(CIP)]Cl_2$ standard stock solution (5.0×10^{-3} mol/L) was prepared by dissolving 207.1 mg $[Ru(bpy)_2(CIP)]Cl_2$ in water and diluting to 50 mL. Ce(IV) working solution (2.0×10^{-4} mol/L) was obtained by dissolving 27.4 mg $(NH_4)_2Ce(NO_3)_6$ (Beijing Xinhua Chemicals Company, Beijing, China) in 250 mL 0.6 mol/L HNO_3. The Ce(IV) solution was freshly prepared.

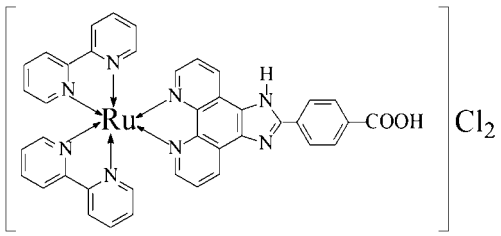

Figure 1. The chemical structure of $[Ru(bpy)_2(CIP)]Cl_2$.

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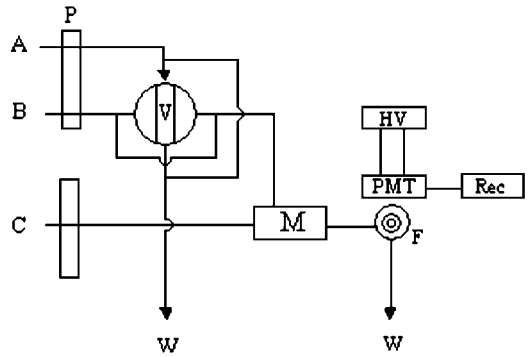

Figure 2. Schematic diagram of the FL–CL analyser. A, Sample; B $[Ru(bpy)_2(CIP)]Cl_2$; C, Ce(IV); P, peristaltic pump; V, sample selection valve with a 30 cm tube; M, mixing well—the PTFE tube length between M and F is 15 cm; F, flow cell (18 μL); W, waste; HV, high voltage; PMT, photomultiplier tube; Rec, recorder.

Apparatus

The FI analyser system used for the determination of OFLX is shown in Fig. 2. A FI-2100 FI system (Beijing Haiguang Instrument Company, China) was used as the FI analyser, which contains two parts, two peristaltic pumps and a 16-way injection valve with a sample loop. PTFE tubing (0.8 mm i.d.) was used to connect all reagents in the flow system. The size of the flow cell was 18 μL. The CL signal was measured using a BPCL ultra-weak luminescence detector (Institute of Biophysics, Academia Sinica, China), which transforms the light signal to an electric signal, and the latter is magnified by a photomultiplier tube. The sensitivity of the photomultiplier tube depends on the value of the negative voltage (0–1000 V) used. Fluorescence and CL spectra were recorded using a RF-5301 PC spectrofluorimeter (Shimadzu, Japan). The kinetic characteristics of the CL system were determined using the BPCL Ultra-CL analyser.

Procedure

As shown in Fig. 2, flow lines (A, B and C) were connected with OFLX, $[Ru(bpy)_2(CIP)]Cl_2$ and Ce(IV) solution, respectively. All solutions were continuously pumped into the manifold at the same flow rate by the two peristaltic pumps (pump rate was 70 revolution (r)/min). A certain amount of OFLX solution was injected into the $[Ru(bpy)_2(CIP)]Cl_2$ carrier stream via the 16-way injection valve every 10 s, and then merged with Ce(IV) solution at M. The mixed solution flowed through a 15 cm PTFE mixing tube and then entered the CL flow cell, giving rise to an intensive CL signal immediately. Peak height was measured for each signal. Calibration graphs were constructed by plotting the relative CL intensity (sample output minus blank) against the concentration of OFLX.

Sample preparation

Six OFLX tablets (Kunshan Double-Crane Pharmaceutical Company, Kunshan, China) were ground into homogenized powder. Then 200 mg powder, corresponding to one tablet, was dissolved with 1.5 mL HCl (0.2 mol/L). The solution was filtered and the residue was washed with water five times. The clear liquid was diluted with water to 100 mL. The two kinds of OFLX eye-drops solutions were prepared by diluting 0.6 mL OFLX eye-drops (1, Wuhan Wujing Medicine company, Wuhan, China; 2, Jiangxi Pingxiang Pharmaceutical Factory, Pingxiang, China) with water to 50 mL respectively. Working solutions were prepared by appropriate dilution of these sample solutions so that the final concentrations were within the linear range.

Results and discussion

Optimization of experimental variables

A series of experiments was conducted to establish the conditions for the production of maximum CL emission for OFLX–$Ru(bpy)_2(CIP)^{2+}$–Ce(IV). The chemical variables, such as concentration of reagents used for the CL reaction, and flow-injection system variables, including flow rate and sample volume, were investigated.

Effect of flow rate

The flow rate of reagents affects the relative CL intensity. In the experiment, every reagent flowed at the same rate. Because the flow rate depends on the pump rate, the effect of pump rate on the relative CL intensity was studied in the range 50–90 r/min. The results showed that the relative CL intensity increased with increasing pump rate. However, at high pump rate, the CL signals became unstable and the reagent consumption was excessive. On balance, 70 r/min was chosen for the pump rate. At this pump rate, the flow rate of reagents was about 2.2 mL/min.

Effect of sample volume

The sample volume is critical; for instance, if sample volume is too small or too large, maximum CL can not be obtained. Sample volume was controlled by a fixed tube length. The effect of the tube length on the relative CL intensity was studied in the range 25–40 cm. The results obtained showed that the CL intensity increased when the length was up to 30 cm, above which the intensity was decreased. Thus, a 30 cm tube, corresponding to a sample volume of 150 μL, was used for further study.

Effect of mixing tube length

The results of the variation of the relative CL intensity with the mixing tube length in the range 15–35 cm showed that the shorter the mixing tube, the larger was the CL intensity. Thus, a 15 cm mixing tube between mixing cell and flow cell was selected for the experiments. The length of the mixing tube used in our study could not be shortened below 15 cm, due to the limitations of the apparatus used.

Effect of acid

A certain volume of acid was added to the Ce(IV) solution, preventing Ce(IV) from hydrolysing. Different acids, HCl, HNO_3 and H_2SO_4, were tested for the determination of OFLX. The greatest CL intensity was obtained using HNO_3. Thus, HNO_3 was chosen for subsequent work.

The influence of HNO_3 concentration in Ce(IV) solution on the relative CL intensity was also examined over the range 0.05–1.5 mol/L. When the HNO_3 concentration was <0.6 mol/L, the relative CL intensity increased with increasing HNO_3 concentration. Then the relative CL intensity kept almost constant when HNO_3 concentration was in the range 0.6–1.0 mol/L. The relative CL

signals slightly decreased in much higher HNO_3 concentrations. Therefore, the chosen concentration of HNO_3 in Ce(IV) solution was 0.6 mol/L.

Effect of Ce(IV) concentration

The effect of Ce(IV) concentration was studied in the range 5.0×10^{-5}–6.0×10^{-4} mol/L. The results showed that the relative CL intensity increased with increasing Ce(IV) concentration, but it was almost constant after the concentration of Ce(IV) reached 2.0×10^{-4} mol/L. Hence, 2.0×10^{-4} mol/L Ce(IV) was chosen.

Effect of $[Ru(bpy)_2(ClP)]Cl_2$ concentration

The relative CL intensity depends on the concentration of $[Ru(bpy)_2(ClP)]Cl_2$, and a study was carried out in the range 1.0×10^{-6}–1.0×10^{-4} mol/L. Although the CL intensity of the system increased with increasing $[Ru(bpy)_2(ClP)]Cl_2$ concentration, the CL intensity of the blank solution also increased. Therefore, taking all factors into consideration, the $[Ru(bpy)_2(ClP)]Cl_2$ concentration of 1.0×10^{-5} mol/L was used for further investigations.

Interference studies

In order to assess the possibility of analytical application of the method under optimum experimental conditions, the effects of some common ions and organic compounds on the determination of 1.0×10^{-6} mol/L OFLX were investigated. A foreign substance was considered to have interference when it showed a determination error of >5%. The results are listed in Table 1. Al^{3+} shows serious interference, but the content of Al^{3+} in pharmaceuticals and biological fluids is low, so it would not effect the determination of OFLX.

Table 1. Effect of the foreign ions or organic substances on CL intensity

Foreign ions or organic substances	Concentration coexisting (1.0×10^{-6} mol/L)	Change of ΔI (%)
Na^+	5000	0.1
K^+	5000	4.7
NH_4^+	5000	1.0
Mg^{2+}	100	−2.2
Ca^{2+}	20	0.4
Ni^{2+}	20	−0.4
Cu^{2+}	20	−4.0
Zn^{2+}	10	1.9
Co^{2+}	10	3.1
Cd^{2+}	5	3.2
Pb^{2+}	2	2.9
Mn^{2+}	2	−1.4
Cr^{3+}	1	−3.5
Al^{3+}	0.1	−13.0
Starch	100[a]	−4.7
β-CD	2[a]	−0.5
Vitamin B_1	0.01[a]	−0.2
Haemoglobin	1[a]	−2.8
Myoglobin	0.02[a]	−2.0
β-Alanine	1	1.4
Glucose	50[a]	−1.2

[a]μg/mL.

Analytical characteristics

Under optimum conditions, the calibration graphs for determination of OFLX were obtained. The linear range was 3.0×10^{-8}–1.0×10^{-5} mol/L. The regression equation was $\Delta I = 6990.3c - 189.26$ ($r = 0.9997$) in the range 3.0×10^{-8}–2.5×10^{-7} mol/L and $\Delta I = 17\,384c - 2131.2$ ($r = 0.9995$) in the range 2.5×10^{-7}–1.0×10^{-5} mol/L, where ΔI is the relative emission intensity and c is the concentration of OFLX.

The detection limit calculated from the standard deviation (SD) of the blank (the reagent blank without OFLX, $n = 11$) (3σ) was 4.2×10^{-9} mol/L. The relative standard deviation (RSD) was 0.31% for 11 determinations of 1.0×10^{-6} mol/L OFLX.

Comparison of the proposed method with previously reported ones is tabulated in Table 2. In this method, the concentration of $[Ru(bpy)_2(ClP)]Cl_2$ used was only 1.0×10^{-5} mol/L. Except for reference (27), the sensitivity of this method is higher than those of other reported methods. The method is simple and 110 samples/h can be analysed.

Analytical application

Analysis of pharmaceutical samples

The proposed method was applied to the determination of OFLX in tablets and eye-drops. The results are summarized in Table 3, which shows that there is no significant difference between the labelled content and that obtained by the proposed method.

Analysis of urine samples

According to a previous report (43), OFLX is mainly excreted in the urine in its original form and the excretion ratio of OFLX in urine is 75.2–87.8% for 24 h. Under the optimum conditions and with the proposed method, the recovery of OFLX in urine was studied, using the calibration method, and the results are given in Table 4. The recoveries were 94.9–100.6%.

Study of pharmacokinetics of OFLX

A healthy volunteer administered 200 mg OFLX tablets, and the real urine samples after 1, 1.5, 3.0, 6.0, 9.0 and 12.0 h were investigated. In order to make the sample concentration of the drug within the linear range, the urine samples were diluted 50-fold with water. Fig. 3 shows the excretion rate of OFLX in urine vs. time. The maximum excretion rate of OFLX appeared at 3 h and the cumulative excretion ratio of OFLX in urine for 12 h was 68.8%. The results were almost consistent with reported reference (44).

Possible CL mechanism

We chose five fluoroquinolones (FQs), including rufloxacin (RFX), norfloxacin (NFLX), ciprofloxacin (CPFX), pazufloxacin (PZFX) and sparfloxacin (SPFX), to compare with OFLX for the sensitizing effect on the CL of $[Ru(bpy)_2(ClP)]Cl_2$–Ce(IV). The structures of the FQs are shown in Fig. 4.

The obtained results showed that PZFX had almost no sensitizing effect on the CL of $[Ru(bpy)_2(ClP)]Cl_2$–Ce(IV). It was found

Table 2. Comparison of this method with previously reported methods

CL system	Linear range (mol/L)	Limit of detection (mol/L)	Reference
$Ru(bipy)_3^{2+}$–Ce(IV)	3.0×10^{-3}–7×10^{-1}[a]	5.5×10^{-9}	25
Peroxynitrous acid	3.0×10^{-7}–3.0×10^{-5}	1.1×10^{-7}	26
Ce(IV)–sulphite	4.0×10^{-2}–4[a]	1.6×10^{-2}[a]	24
$KMnO_4$–Na_2SO_3–Tb(III)	1.0×10^{-9}–1.0×10^{-6}	5.6×10^{-10}	27
Soluble manganese(IV)–sulphite	1.0×10^{-7}–8.0×10^{-6}	5×10^{-8}	28
$Ru(bpy)_2(CIP)^{2+}$–Ce(IV)	3.0×10^{-8}–1.0×10^{-5}	4.2×10^{-9}	This paper

[a]μg/mL.

Table 3. Results of determination for OFLX in tablets and eye-drops ($n = 8$)

Sample	Label (mg)	Present method (mg)	RSD (%)
Tablets	100.0	103.2, 98.9, 99.3, 96.0, 101.6, 96.9, 98.1, 93.4	3.1
Eye-drops 1	15.0	14.9, 14.8, 14.2, 14.8, 14.3, 14.7, 13.8, 14.9	3.6
Eye-drops 2	15.0	14.5, 15.2, 14.5, 14.4, 14.4, 14.9, 14.9,15.2	2.3

Table 4. Recovery study of OFLX in urine samples ($n = 5$)

Sample	Added ($\times 10^{-7}$ mol/L)	Found ($\times 10^{-7}$ mol/L)	Recovery (%)	RSD (%)
Urine 1	2.00	2.01	100.6	5.2
	3.00	2.92	97.4	4.4
	4.00	4.01	100.3	2.8
Urine 2	1.00	0.949	94.9	2.3
	2.00	1.92	96.1	2.9
	3.00	2.99	99.7	3.1

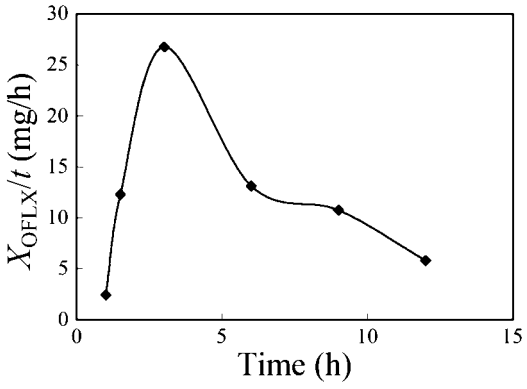

Figure 3. Excretion rate of OFLX in urine.

that PZFX has only one substituent differing from OFLX, which is the 7-substituent. According to the reported literature (45), $Ru(bipy)_3^{2+}$ CL is very sensitive to compounds which contain a secondary or tertiary amine. OFLX has tertiary amine in the piperazine moiety, while PZFX has neither tertiary nor secondary amines. It therefore seems that the CL phenomenon is related to the secondary or tertiary amine, which is similar to other reports in the literature (25, 45).

The sensitizing effect of SPFX is very weak, but other FQs are similar to OFLX, which causes a remarkable enhancement to the CL of $[Ru(bpy)_2(CIP)]Cl_2$–Ce(IV). Hence, the proposed method can also be employed to determine the other three FQs, and their analytical parameters are listed in Table 5.

The kinetic curves of CL systems containing different FQs are plotted in Fig. 5. It can be seen that the reaction of the $Ru(bpy)_2(CIP)^{2+}$–Ce(IV)–FQs system was very rapid and the maximum intensity was reached at 3 s for OFLX and RFX and at 4 s for CPLX and NFLX.

The CL spectra (Fig. 6) show that the emission peaks were located at 617–624 nm in the absence and presence of FQs, being consistent with the fluorescence spectrum of $[Ru(bpy)_2(CIP)^{2+}]$ (Fig. 7). This therefore indicates that the final emitter of the system is $[Ru(bpy)_2(CIP)^{2+}]^*$.

The overall CL reaction pathways may be as follows:

$$Ru(bpy)_2(CIP)^{2+} + Ce(IV) \rightarrow Ru(bpy)_2(CIP)^{3+} + Ce(III)\ \text{(oxidation)} \quad (1)$$

$$FQ + Ce(IV) \rightarrow FQ^{\cdot+} + Ce(III)\ \text{(oxidation)} \quad (2)$$

$$FQ^{\cdot+} \rightarrow FQ^{\cdot} + H^+\ \text{(deprotonation)} \quad (3)$$

$$Ru(bpy)_2(CIP)^{3+} + FQ^{\cdot} \rightarrow [Ru(bpy)_2(CIP)^{2+}]^* + \text{FQ fragments (electron transfer)} \quad (4)$$

$$[Ru(bpy)_2(CIP)^{2+}]^* \rightarrow Ru(bpy)_2(CIP)^{2+} + h\nu\ \text{(light emission)} \quad (5)$$

The differences in CL activity between different FQs can be explained by considering their structures. Oxidation of FQs can form electron-deficient radicals, and the more stable the radical, the more efficient is the CL. Since an electron-deficient radical can be more stabilized by the alkyl of a tertiary amine, a tertiary amine is more active than a secondary amine (45). The piperazine moiety of FQs structures contains different substituents. It is guessed that $FQ^{\cdot}$, in which there is piperazine ring directly connected to the aromatic ring, is more stable than $FQ^{\cdot}$ formed from PZFX. But SPFX is not sensitive, possibly because the fluorine in the 8-substitution position shows electron-withdrawing property, which will destabilize the radical intermediate. Also, OFLX and

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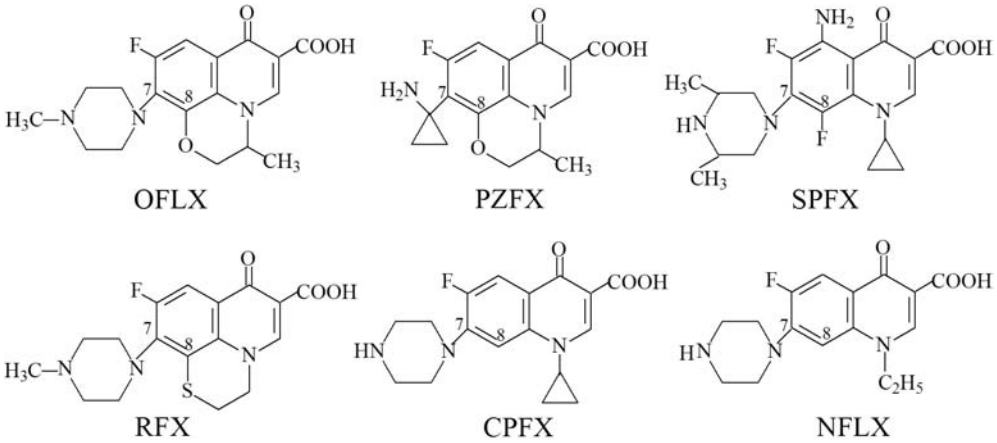


Figure 4. Chemical structures of FQs.

Table 5. Calibration curves and detection limits of FQs

FQs	Linear range (mol/L)	Regression equation	*r*	Detection limit (mol/L)
CPFX[a]	4.0×10^{-8}–1.0×10^{-5}	$\Delta I = 117.22 \times 10^8 c - 1920.9$	0.9981	4.8×10^{-9}
NFLX[a]	4.0×10^{-8}–4.0×10^{-6}	$\Delta I = 992.02 \times 10^7 c - 771.8$	0.9989	5.1×10^{-9}
	4.0×10^{-6}–1.0×10^{-5}	$\Delta I = 152.39 \times 10^8 c - 22\,317.1$	0.9994	
RFX[b]	2.0×10^{-8}–4.0×10^{-6}	$\Delta I = 977.30 \times 10^7 c - 885.3$	0.9984	3.3×10^{-9}
	4.0×10^{-6}–1.0×10^{-5}	$\Delta I = 136.74 \times 10^8 c - 17\,304.0$	0.9967	

Conditions: pump rate, 70 r/min; flow and injection time,10 s, fixed quantity tube length, 30 cm; $Ru(bpy)_2(ClP)Cl_2$, 1.0×10^{-5} mol/L.
[a]Mixing tube length, 20 cm; HNO_3, 0.8 mol/L; Ce(IV), 2.0×10^{-4} mol/L.
[b]Mixing tube length, 15 cm; HNO_3, 0.6 mol/L; Ce(IV), 4.0×10^{-4} mol/L.

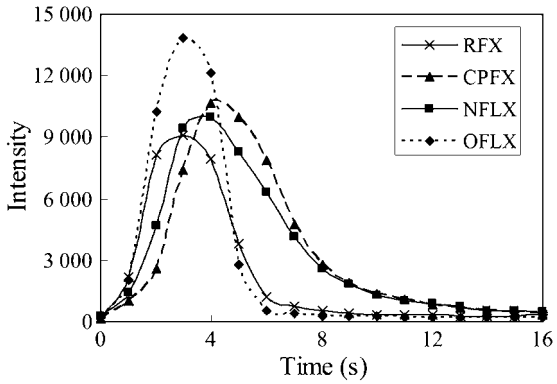


Figure 5. Kinetic curves for the CL systems.

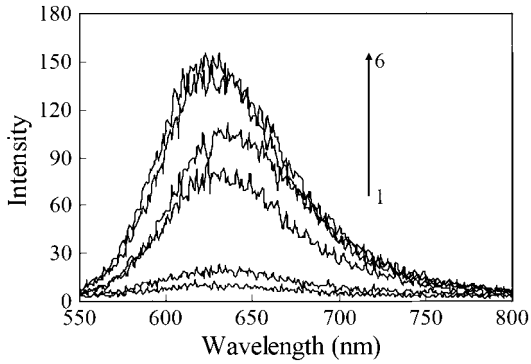
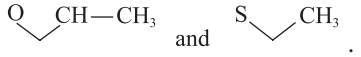


Figure 6. CL spectra of FQs–Ce(IV)–$[Ru(bpy)_2(ClP)]Cl_2$ system: 1, Ce(IV)–$[Ru(bpy)_2(ClP)]Cl_2$; 2, 1 + SPFX; 3, 1 + NFLX; 4, 1 + CPFX; 5, 1 + RFX; 6, 1 + OFLX.

RFX have more intense signals, probably due to the electron-donating effect of the 8-substituents:



Conclusions

A simple, rapid and sensitive FI–CL method is described for the determination of OFLX, based on the sensitizing effect of OFLX on the CL of $Ru(bpy)_2(ClP)^{2+}$–Ce(IV). The proposed method can be successfully applied to the determination of OFLX in pharmaceuticals and urine.

Acknowledgements

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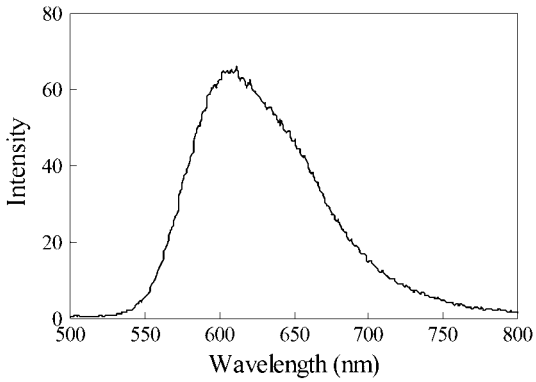

Figure 7. Fluorescence spectrum of $[Ru(bpy)_2(CIP)]Cl_2$.

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