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Flow-Injection Chemiluminescence Determination of Fleroxacin in Pharmaceutical Preparations and Human Urine

Hanwen Sun[1] and Liqing Li[2]

[1]College of Chemistry and Environmental Science, Hebei University, Key Laboratory of Analytical Science and Technology of Hebei Province, Baoding, 071002, China
[2]Department of Chemistry, Taishan University, Taian, 271021 China

Abstract

Quality control of drug fleroxacin dosage, its monitoring in biological fluids, and research of drug's metabolism and action are an important analytical task. A new chemiluminescence (CL) reaction system was established for the determination of fleroxacin (FLX). The trivalence dysprosium-sensitized CL emission mechanism was investigated by comparing the fluorescence emission with CL spectra. The CL spectra of FLX-$KMnO_4$-$Na_2S_2O_3$-$H_6P_4O_{13}$ system are from the narrow characteristic emission of Dy^{3+} at 482 and 578 nm ($^4F_9 \rightarrow {}^6H_{15/2}$, $^4F_9 \rightarrow {}^6H_{13/2}$) through the energy transfer from the excited SO_2* to analyte, followed by intramolecular energy transfer from analyte* to Dy^{3+}. The optimum conditions for CL emission were investigated and optimized. The relationships between the relative CL intensity and the concentration of the studied analyte have good linearity. The detection limit for FLX was 3.0×10^{-10} g/mL. The relative standard deviation is 2.0% for 11 determinations of FLX at 2.0×10^{-6} g/mL. The proposed CL system has been successfully applied for the determination of FLX in the injections and urine sample with satisfactory result. .

Keywords: Chemiluminescence; Fleroxacin; Pharmaceutical preparations; Human urine

Introduction

Fleroxacin [FLX, 6,8-difluoro-1-(2-fluoroethyl)-1,4-dihydro-7-(4-methyl-1- piperazinyl)-4-oxo-3-quinoline carboxylic acid] is a new fluoroquinolone antibiotic that exhibits strong bactericidal activity against a wide range of Gram-negative and Gram-positive bacteria [1]. Cullmann et al. reviewed the chemistry, microbiology, toxicology, pharmacokinetics, clinical efficacy and safety of FLX [2]. The mechanism of action of FLX is based primarily on the inhibition of bacterial DNA topoisomerase II (DNA gyrase). Quality control of drug dosage, its monitoring in biological fluids, and research of drug's metabolism and action are an important analytical task. Therefore, it is necessary to establish sensitive analytical technique.

Several methods have been described for the determination of FLX either in pure form, in dosage forms or in biological fluids [3-10]. High-performance liquid chromatography (HPLC) with fluorescence detection has been developed for the measurement of FLX in rat plasma using a solid-phase extraction column [11], and FLX in serum [12]. Capillary electrophoresis (CE) and HPLC have the advantage of high separation capability suitable for components determination, and disadvantage of lower sensitivity.

The chemiluminescence (CL) method shows the advantages of simplicity, rapidity and high sensitivity, and has been applied extensively for the analysis of pharmaceutical compounds [13,14]. Chemiluminescence sensors are important tools in analytical chemistry due to their high sensitivity and selectivity [15-18]. A critical review was presented for the use of acidic solutions of potassium permanganate to generate CL during the oxidation of both organic compounds and inorganic species [19]. The CL reactions of potassium permanganate and reducer have been studied extensively. Among them, sodium thiosulfate is a classical reducer and has been used with potassium permanganate to detect some pharmaceutical compounds, but CL emission from the redox reaction of potassium permanganate and sodium thiosulfite is not significant enough. For cerium(IV)-sulfite CL system, the reduction–oxidation reaction between Ce(IV) and sulfite shows a weak peak. Recently, Chen and Fang reviewed flow injection technique for biochemical analysis with CL detection in acidic media [20]. New recently, a new CL method is reported for the determination of fluoroquinolone derivatives based on the enhancement of CL of luminol-hydrogen peroxide-gold nanoparticles system by fluoroquinolones [21]. The detection limits of the reported methods for the determination of FLX were at 10^{-9}–10^{-7} g/mL levels.

The main purpose of this work is to develop a new Dy^{3+}-sensitized CL system for the determination of FLX. The proposed method was applied for the determination of FLX in the injectable and urine samples with satisfactory result. The CL mechanism was also described.

Experimental Section

Chemicals and solution

All chemicals used were of analytical-reagent grade. Deionized water was used throughout. FLX was purchased from Institute of Medicinal Biotechnology Beijing, China). The Stock standard solution (5.0×10^{-4} g/mL) for FLX was prepared by dissolving 25.00 mg analyte in 1.5 mL 0.1 M sodium hydroxide, and diluting with deionized water to 50 mL, respectively. The more diluted solutions were freshly prepared by diluting the stock solution with deionized water.

A Dy^{3+} stock solution, 1×10^{-2} M, was prepared by dissolving 373 mg Dy_2O_3 in 15.0 mL HCl (11.6 M) at 95°C, evaporating the solution to be almost dry, then diluting it to 100 mL with water. Stock $KMnO_4$ solution (5×10^{-2} M) and $Na_2S_2O_3$ solution (2×10^{-3} M) were prepared daily and diluted as required. The working solutions of $Na_2S_2O_3$, H_2SO_4, $H_6P_4O_{13}$, HNO_3 and HCl were prepared daily and diluted as required.

***Corresponding author:** Hanwen Sun, College of Chemistry and Environmental Science, Hebei University, Key Laboratory of Analytical Science and Technology of Hebei Province, Baoding, 071002, China, E-mail: hanwen@hbu.edu.cn

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Apparatus

The FI system, as shown in Figure 1, was a MPI-B flow-injection chemiluminescence analysis system (Xi'an Remex Electronic science-tech Co. Ltd., Xi'an, China) consisted of two peristaltic pumps working at a constant flow rate (30 rpm) and a six-way injection valve with a sample loop (120μL), which is automatically operated by a computer equipped with a software for operation system of MPI-B flow injection analysis. The flow cell is a twistied glass tube in order to produce a large surface area exposed to the adjacent photomultiplier tube (PMT) (Hamamatsu, Japan).

PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow system. The signal from the CL reaction was recorded. Fluorescence spectra were recorded with RF-5301PC spectrofluorometer (Shimadzu, Japan) for the study of the fluorescence characteristics.

Procedure

The injection sample of FLX was made of 20 bottles of FLX injection selected from same group randomly. The working solutions were directly diluted with water. Human urine was kindly provided by healthy volunteers. No further pre-treatment was required for urine samples.

As shown in Figure 1, all solutions were continuously pumped into the manifold. A 120μL mixture of analyte solution and $Na_2S_2O_3$ solution was injected into a mixed stream of $KMnO_4$ and Dy^{3+} solutions. The mixed solution was transferred into the CL flow cell, and gave rise to an intensive CL signal immediately. The CL signal produced in the flow cell was recorded. Calibration graphs were constructed by plotting the intensity (peak height) of the CL signal versus the concentration of analyte.

Results and Discussion

Choice of sensitizers and CL system

Both $KMnO_4$-$S_2O_3^{2-}$ and MnO_4-$S_2O_3^{2-}$-FLX systems could only produce weak CL emission, respectively. The effects of various fluorescence compounds, such as rhodamine 6G, rhodamine B, eosin and fluorescein, on CL emission were investigated. No enhancing effect was observed clearly. Based on the fluorescence properties of lanthanide ions, La^{3+} and Lu^{3+} (no emitting fluorescence), Gd^{3+} (lightly emitting fluorescence), Sm^{3+} , Eu^{3+}, Dy^{3+} and Tb^{3+} (highly emitting fluorescence), and Pr^{3+}, Nd^{3+} , Ho^{3+}, Er^{3+}, Tm^{3+}, Yb^{3+} (low fluorescence efficiency) were tested as sensitizers for the MnO_4-$S_2O_3^{2-}$-FLX CL system, respectively. The experimental results indicated that Dy^{3+} and Tb^{3+} enhanced obviously the CL signals of $KMnO_4$-$S_2O_3^{2-}$-FLX system. The further test showed that the intensity of Dy^{3+}-sensitized chemiluminescence signal was higher than that of Tb^{3+}-sensitized chemiluminescence signal for $KMnO_4$-$S_2O_3^{2-}$-FLX system.

The effects of Dy^{3+} concentration on the CL intensity for the system were investigated in the range of 1×10^{-4}-6×10^{-4} M. The CL intensity increased obviously with the increase of Dy^{3+} concentrations in the range of 1×10^{-4}-4×10^{-4} M, and decreased above 4×10^{-4} M. The Dy^{3+} concentration of 4×10^{-4} M was selected for FLX-$KMnO_4$-$Na_2S_2O_3$ system with the maximum CL intensity.

Effect of sample volume and flow rate on detection

As shown in Figure 1, when the mixed solution flowed into the cell, the CL reaction took place. The role of sample volume and flow rate is critical, for instance, if sample volume and flow rate were too small or too large, CL maximum could not be obtained. Whan the injected sample volume of 120μL and flow rate of 3.0 mL/min for all solutions were used, the highest emission was obtained along with greater precision and economy in the use of reagents.

Effect of acidic medium on detection

The kind and concentration of the acid used in the reaction has a very significant influence on the CL emission intensity. Therefore several acids, such as HCl, H_2SO_4, HNO_3, H_3PO_4 and $H_6P_4O_{13}$, were added in FLX–$KMnO_4$–$Na_2S_2O_3$ solution to test the effect of acidic medium on the CL signal, respectively. The highest and stable emission was observed in $H_6P_4O_{13}$ medium for FLX–$KMnO_4$–$Na_2S_2O_3$ system, and the optimal concentration was 1×10^{-5} M.

Effect of $KMnO_4$ concentration on detection

In this CL system, $KMnO_4$ was used as the oxidant. The $KMnO_4$ concentration influences the sensitivity. Therefore, the dependence of the $KMnO_4$ concentration on the CL intensity was investigated for 1.0×10^{-6} g/mL analyte. The CL intensity increased with increasing $KMnO_4$ concentration from 0.5×10^{-4} to 2.5×10^{-4} M, and decreased obviously in range of 2.5×10^{-4}–1.0×10^{-3} M. The $KMnO_4$ concentration of 2.5×10^{-4} M was selected with the maximum CL intensity.

Effect of sodium thiosulfate concentration on detection

The effect of sodium thiosulfate concentration over the range of 5×10^{-5}–5×10^{-4} M on CL emission was examined for 1.0×10^{-6} g/mL analyte. The maximum CL emission was obtained under the sodium thiosulfate concentration of 7.5×10^{-5} M.

Interference studies

The influence of some common excipients used in drugs was investigated for the determination of 4.0×10^{-7} g/mL analyte by

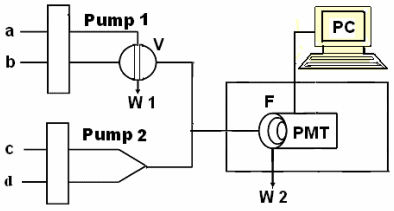

Figure 1: Schematic diagram of flow injection CL analysis system V—sampling inlet valve; F—flowing cell: a flat spiral-coiled colorless glass tube (i.d. 1.0 mm, total diameter of the flow cell 3 cm, without gas between loops); PMT—photomultiplier tube; W—waste; a—analyte solution; b—$Na_2S_2O_3$ solution; c—acidic $KMnO_4$ solution; d—Dy^{3+} solution.

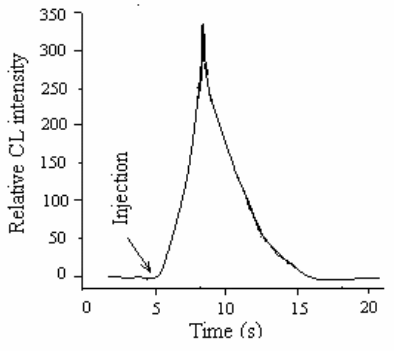

Figure 2: CL intensity–time profile of of Dy^{3+}-FLX -$KMnO_4$-$Na_2S_2O_3$ system $KMnO_4$, 2.5×10^{-4} M; $Na_2S_2O_3$, 7.5×10^{-5} M; Dy^{3+}, 4×10^{-4} M; $H_6P_4O_{13}$, 2×10^{-5} M; FLX: 1×10^{-5} g/mL.

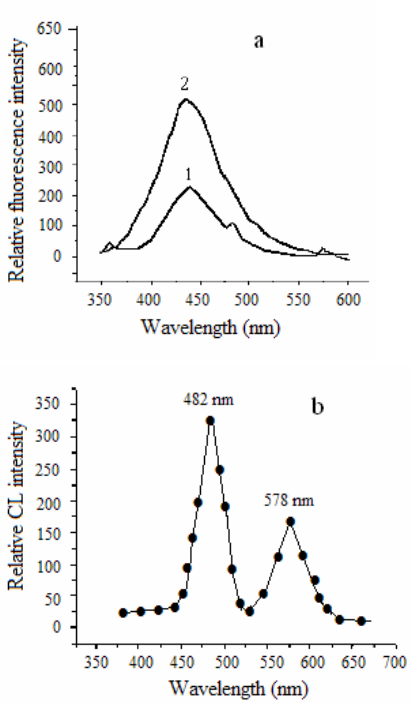

Figure 3: Fluorescence emission spectra of Dy^{3+}-FLX(a1) and FLX (a2) and CL spectra of Dy^{3+}-FLX -$KMnO_4$-$Na_2S_2O_3$(b) a: λ_{ex}=303 nm; Dy^{3+}, 2×10^{-4} mol L^{-1}; FLX, 2×10^{-7}g/mL; b: $KMnO_4$, 2.5×10^{-4} M; $Na_2S_2O_3$,7.5×10^{-5} M; Dy^{3+}, 4×10^{-4} M; $H_6P_4O_{13}$, 2×10^{-5} M; FLX: 1×10^{-5} g/mL.

comparing with the CL emissions obtained using analyte solution alone or analyte with foreign species added. A substance was considered no interference if the variation of the CL intensity was $<\pm5\%$. The results indicate that 100-fold magnesium stearate, sucrose, dextrin, galactose, fructose, starch, lactose, 60-fold glucose, 50-fold sodium benzoate, polyglycol, and 20-fold sodium citrate did not interference for the determination of 4.0×10^{-7} g/mL FLX.

Kinetic characteristics of CL reaction

The chemiluminescence kinetic characteristics of the reactions of Dy^{3+}-FLX-$Na_2S_2O_3$-$KMnO_4$-$H_6P_4O_{13}$ system were investigated The CL intensity–time profile of the system is presented in Figure 2.

It was found that the reaction rate in solution was very fast, from reagent mixing to peak maximum only 3 s was needed for Dy^{3+}-FLX-$Na_2S_2O_3$-

$KMnO_4$-$H_6P_4O_{13}$ system, and it took 9 s for the signal to return to zero again.

Analytical performance of CL system

The proposed CL method can process up to 60 samples per hour. Under the optimum conditions described above, the linearity and relative standard deviation (RSD) for detection of **FLX** were investigated. The calibration graph was consists of five parts for FLX in order to improve the veracity. The experimental results are listed in Table 1.

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a height three times of the level of baseline noise, and the limit of quantification (LOQ) was calculated as the sample concentration that produces a peak with a height ten times the baseline noise [22, 23]. The LOD was 3.0×10^{-10} g/mL and LOQ was 1.2×10^{-9} g/mL for the first equations of FLX. The relative standard deviation was 1.9% for 11 determinations of 6.0×10^{-8} g/mL of FLX. The proposed method has lower LOD than UV spectrophotometry[3], fluorescence spectrometry [4,5], voltammetric method [7] and HPLC [9-12] as well as luminol-hydrogen peroxide-gold nanoparticles CL method [21]. It is indicated that the proposed CL system has good linearity, higher sensitivity and precision.

CL mechanism

The chemiluminescence intensity of $KMnO_4$-$Na_2S_2O_3$-$H_6P_4O_{13}$ system is very weak because of the low luminescence efficiency of SO_2^*[23]. By introducing a fluorophore whose absorption falls in the emission range of the excited sulfur dioxide (300–450 nm) [24], the CL intensity is usually enhanced through energy transfer from SO_2^* to the fluorophore [25], $Na_2S_2O_3$ in acidic medium react to produce HSO_3^-, based on this, Dy^{3+} or analyte was added to the CL system of $KMnO_4$-$Na_2S_2O_3$, respectively, but no notable increase in the CL intensity could be observed. However, when Dy^{3+} and analyte were added together to the CL system of $KMnO_4$-$Na_2S_2O_3$, the CL intensity was greatly enhanced.

In order to gain a better understanding of the nature of the CL enhancement, we examined the CL spectra of Dy^{3+}-$KMnO_4$-$Na_2S_2O_3$-FLX system by a series of interference filters and the fluorescence emission spectra of the system, as shown in Figure 3.

The native fluorescence emission of FLX shows broad peak centers at 438 nm and 445 nm, respectively. When mixing with Dy^{3+}, this wide emission band decreases in intensity greatly while the narrow emission bands of the Dy^{3+} appear at 482 and 578 nm, corresponding to the transitions of the Dy^{3+}, $^4F_9\rightarrow{}^6H_{15/2}$ and $^4F_9\rightarrow{}^6H_{13/2}$, respectively [26], which implies that the intramolecular energy transfer has occurred between analyte and the Dy^{3+} [27-29]. Meanwhile, it can be concluded that the Dy^{3+}-analyte complex has been formed.

As shown in Figure 3b, the sensitized CL spectra of Dy^{3+}-$KMnO_4$-$Na_2S_2O_3$-FLX system are located at 482 and 578 nm, which is the characteristic fluorescence spectrum of dysprosium [23], indicating clearly that the excited Dy^{3+} is the emitter, and there must be energy transfers in the CL systems. Since Dy^{3+} forms the chelate with analyte,

Regression equation	Correlation coefficient	Linear range (g/mL)
I=1.3C +13.0	0.9979	1.0×10^{-9}–1.0×10^{-8}
I=9.9 C+13.7	0.9980	1.0×10^{-8}–1.0×10^{-7}
I=54.2C +57.4	0.9978	1.0×10^{-7}–1.0×10^{-6}
I=331.6C +201.5	0.9972	1.0×10^{-6}–1.0×10^{-5}
I=499.2C +3165.3	0.9984	1.0×10^{-5}–6.0×10^{-5}

Table 1: Regression equation and RSD for determinations of FLX.

Content ($\times10^{-7}$g/mL)	Added ($\times10^{-7}$g/mL)	Found ($\times10^{-7}$g/mL)	Recovery %, n=7
0.33	0.1	0.44	110.0
	0.3	0.65	106.7
	0.5	0.85	104.0
2.65	2.0	4.54	99.0
	4.0	6.60	103.5
	6.0	8.85	104.9

Table 2: Recovery experiments for FLX.

Time (h)	Content ($\times10^{-5}$ g/mL)	Added ($\times10^{-5}$ g/mL)	Found ($\times10^{-5}$ g/mL)	Recovery (%)	RSD n=5 (%)
1	*	0.002	0.0021	105	2.2
2	1.0	2.0	3.05	103	1.8
4	10.8	10.0	20.7	99.0	1.7
8	7.8	5.0	12.7	98.0	1.6
12	7.2	5.0	12.2	100	1.8
* not detected					

Table 3: Determination of FLX in urine samples.

these complexes absorb the energy at the characteristic wavelength of the organic ligand and emit radiation at the characteristic wavelength of the lanthanide due to an energy transfer from the quinolone ligand to the emitting energy level of the metal ion.

An intermolecular energy transfer takes place from SO_2^* to the ligand (analyte) in the chelate (Dy^{3+}-FLX) produced in the reaction process. Then, through intramolecular energy transfer from the ligand* to Dy^{3+}, Dy^{3+*}-ligand is formed, followed by the narrow characteristic emission of Dy^{3+*}. The mechanism stated above can be expressed as follows:

$$S_2O_3^{2-} + H^+ \rightarrow HSO_3^-$$

$$MnO_4^- + HSO_3^- \rightarrow MnO_4^{2-} + HSO_3^*$$

$$2\ HSO_3^* \rightarrow S_2O_6^{2-} + 2\ H^+$$

$$S_2O_6^{2-} \rightarrow SO_4^{2-} + SO_2^*$$

$$SO_2^* + [Dy\text{-}FLX]^{3+} \rightarrow SO_2 + [Dy\text{-}FLX^*]^{3+}$$

$$[Dy\text{-}FLX^*]^{3+} \rightarrow [Dy^*\text{-}FLX]^{3+}$$

$$[Dy^*\text{-}FLX]^{3+} \rightarrow [Dy\text{-}FLX]^{3+} + h\nu\ (482\ nm, 578\ nm)$$

Pharmaceutical analysis

The injectable consists of FLX, lactic acid and glucose. Labeled content of FLX is 400 mg/100 mL for the injectables (batch numbe: 040710205 and 0412231). In order to evaluate the validity of the proposed method for the determination of FLX, recovery studies were carried out on the injectable to which known amounts of analyte were added. The spiked injectable sample was diluted by 100 fold, and determined using the fifth regression equation in Table 1. The recovery is given in Table 2.

The FLX content of 412mg/100mL and 409mg/100mL in the injectables was obtained, respectively, and the relative standard deviation (RSD, n=7) was 1.9%. There was no significant difference between the labeled contents and the results obtained by the proposed method. Furthermore, the results agree well with the content (408mg/100mL and 407mg/100mL) obtained by CE method [30].

Urine sample analysis

Following oral administration, FLX is rapidly and completely absorbed from gastrointestinal tract and found in body tissues, fluids, blood and urine. After an oral dose of 400 mg a maximum plasma concentration of approximately 5×10^{-6} g/mL is reached in approximately 1–2 h [3]. Urine is the major route of excretion of FLX (50–60% of the dose). The healthy volunteer was treated with an oral administration of 200 mg FLX tablet. After oral administration of FLX the urine samples were collected at 1, 2, 4, 8, and 12 h, respectivly. The urine collected before dosing was employed as a blank. In order to make the sample concentration of the drug within the linear range of determination, the urine samples were diluted by 10 fold and analyzed by the standard addition method. The results obtained are given in Table 3.

The highest content of FLX was observed in the urine selected at 4 h after oral administration. The relative standard deviation of peak areas was used to express intra- and inter-day precision. The blank urine samples spiked at 2×10^{-7} g/mL level were analyzed in four replicates on a single day. An intra-day precision (n=4) of 2.5% and an inter-day precision (n=4) of 3.5% were achieved.

Conclusions

Dy^{3+}-enhanced chemiluminescence system was developed for determination of fleroxacin. An intermolecular energy transfer takes place from SO_2^* to the ligand (analyte) in the chelate (Dy^{3+}-FLX) produced in the reaction process. Then through intramolecular energy transfer from the ligand* to Dy^{3+}, Dy^{3+*}-ligand is formed, followed by the narrow characteristic emission of Dy^{3+*}. The CL spectra of Dy^{3+}-$KMnO_4$-$S_2O_3^{2-}$-analyte systems are from the narrow characteristic emission of Dy^{3+*} at 482 and 578 nm. The proposed enhanced CL systems have good linearity, higher sensitivity, precision and potential capability for residue analysis of studied analytes in foods and biological samples. FLX metabolites need to be investigated.

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