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

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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-KMnO₄-Na₂S₂O₃-H₃PO₄ system are from the narrow characteristic emission of Dy³⁺ at 482 and 578 nm (F⁺ → H₁⁹⁺→ F⁻ → H₁⁶⁻) through the energy transfer from the excited SO₄²⁻ to analyte, followed by intramolecular energy transfer from analyte* to Dy³⁺. 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 2.0×10⁻⁹ 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 thiosulfate 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 10⁻⁹-10⁻¹⁰ g/mL levels.

The main purpose of this work is to develop a new Dy³⁺-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 Dy³⁺ stock solution, 1×10⁻³ M, was prepared by dissolving 373 mg Dy₂O₃ 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 KMnO₄ solution (5×10⁻² M) and Na₂S₂O₃ solution (2×10⁻³ M) were prepared daily and diluted as required. The working solutions of Na₂S₂O₃, H₂SO₄, H₃PO₄, HNO₃ and HCl were prepared daily and diluted as required.

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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 twisted 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 Na₂S₂O₃ solution was injected into a mixed stream of KMnO₄ and Dy³⁺ 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 KMnO₄-S₂O₃²⁻ and MnO₂-S₂O₃²⁻-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, La³⁺ and Lu³⁺ (no emitting fluorescence), Gd³⁺ (lightly emitting fluorescence), Sm³⁺, Eu³⁺, Dy³⁺ and Tb³⁺ (highly emitting fluorescence), and Pr³⁺, Nd³⁺, Ho³⁺, Er³⁺, Tm³⁺, Yb³⁺ (low fluorescence efficiency) were tested as sensitizers for the MnO₂-S₂O₃²⁻-FLX CL system, respectively. The experimental results indicated that Dy³⁺ and Tb³⁺ enhanced obviously the CL signals of KMnO₄-S₂O₃²⁻-FLX system. The further test showed that the intensity of Dy³⁺-sensitized chemiluminescence signal was higher than that of Tb³⁺-sensitized chemiluminescence signal for KMnO₄-S₂O₃²⁻-FLX system.

The effects of Dy³⁺ 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 Dy³⁺ concentrations in the range of 1×10⁻⁴-4×10⁻⁴ M, and decreased above 4×10⁻⁴ M. The Dy³⁺ concentration of 4×10⁻⁴ M was selected for FLX-KMnO₄-Na₂S₂O₃ 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. When 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, H₂SO₄, HNO₃, H₃PO₄ and H₆P₄O₁₃, were added in FLX-KMnO₄-Na₂S₂O₃ solution to test the effect of acidic medium on the CL signal, respectively. The highest and stable emission was observed in H₆P₄O₁₃ medium for FLX-KMnO₄-Na₂S₂O₃ system, and the optimal concentration was 1×10⁻⁵ M.

**Effect of KMnO₄ concentration on detection**

In this CL system, KMnO₄ was used as the oxidant. The KMnO₄ concentration influences the sensitivity. Therefore, the dependence of the KMnO₄ concentration on the CL intensity was investigated for 1.0×10⁻⁶ g/mL analyte. The CL intensity increased with increasing KMnO₄ 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 KMnO₄ 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 5×10⁻⁵-3×10⁻⁴ 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 7.5×10⁻³ 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
reagent mixing to peak maximum only 3 s was needed for Dy\(^{3+}\)-FLX-Na\(_2\)S\(_2\)O\(_3\)-

...intensity–time profile of the system is presented in Table 1.

investigated. The calibration graph was consists of five parts for FLX

times the baseline noise \[22, 23\]. The LOD was 3.0×10\(^{-10}\) g/mL and LOQ

as the sample concentration that produces a peak with a height ten

of baseline noise, and the limit of quantification (LOQ) was calculated

calculating the LOQ. 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\(^{-10}\) 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 K\(_2\)MnO\(_4\)-Na\(_2\)S\(_2\)O\(_3\)-H\(_6\)P\(_4\)O\(_{13}\) 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\(_2\)S\(_2\)O\(_3\) in acidic medium react to produce

HSO\(_3^-\), based on this, Dy\(^{3+}\) or analyte was added to the CL system of K\(_2\)MnO\(_4\)-Na\(_2\)S\(_2\)O\(_3\), 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 K\(_2\)MnO\(_4\)-Na\(_2\)S\(_2\)O\(_3\), 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\(^{3+}\)-K\(_2\)MnO\(_4\)-Na\(_2\)S\(_2\)O\(_3\)-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, 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\(^{3+}\)-K\(_2\)MnO\(_4\)-Na\(_2\)S\(_2\)O\(_3\)-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,
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 intramolecular energy transfer takes place from SO₄²⁻ to the ligand (analyte) in the chelate ([Dy⁺⁻FLX]) produced in the reaction process. Then, through intramolecular energy transfer from the ligand to Dy⁺⁺, Dy⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓→…\n
\[ S_2O_6^{2-} \rightarrow SO_4^{2-} + 2H^+ \]

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

Conclusions

Dy⁺⁺-enhanced chemiluminescence system was developed for the determination of fleroxacin. An intramolecular energy transfer takes place from SO₄²⁻ to the ligand (analyte) in the chelate ([Dy⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓→…\n
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