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Investigation on the Micelle-Sensitized Ce (IV) - Lornoxicam-Rh B Chemiluminescence System and its Application

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Abstract Based on the micelle synergism mechanism, a simple and sensitive flow injection chemiluminescence (FI-CL) method for the assay of lornoxicam was described. The CL signal generated from the reaction of Ce (IV) with lornoxicam in acidic solution was very weak, while the interfusion of sodium dodecyl benzene sulfonate (SDBS) resulted in a highly CL intensity. Under the optimum experimental conditions, the CL intensity was proportional to lornoxicam concentration over the range 1.0×10^{-10}–7.3×10^{-8} g/mL with a detection limit of 4.9×10^{-11} g/mL (3σ). The relative standard deviation for 11 replicate measurements of 3.0×10^{-9} g/mL of lornoxicam was 1.9%. The proposed method was successfully applied for the assay of lornoxicam in pharmaceuticals, human serum and urine with excellent recovery. The possible mechanism of CL reaction was also discussed briefly.

Keywords Chemiluminescence · Lornoxicam · Ce (IV) · Sodium dodecyl benzene sulfonate · Rh B

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Introduction

Lornoxicam (LXM), chemically named 6-chloro- 4-hydroxy- 2-methyl-N-2- pyridinyl- 2H-thieno[2,3-e]-1,2-thiazine-3-carboxamide 1,1-dioxide (Fig. 1), is a novel non-steroidal anti-inflammatory drug (NSAIDs) with marked analgesic properties [1, 2]. LXM, belongs to the chemical class oxicam, is prescribed for osteoarthritis, rheumatoid arthritis, acute lumbar-sciatica conditions and postoperative pain management [3, 4]. The pharmacological actions of LXM are attributed to the nonselective inhibition of cyclooxygenase-1(COX-1) and cyclooxygenase-2 (COX-2) that are involved in the synthesis of inflammatory prostaglandins [5–8]. It is distinguished from established oxicams by a relatively short elimination half life, which may be advantageous from a to lerability stand point [9].

Because of the recent interest for use of LXM in the treatment of various post operative pain and rheumatoid arthritis (RA), the therapeutic important of this drug requires the development of simple, rapid, sensitive and industrial quality control and clinical monitoring. For its measurement, several techniques have been published for the assay of LXM in pharmaceutical formulations and biological fluids. These include UV–vis spectrophotometry and spectrofluorometry [10–14], electrochemistry [15, 16], thin layer chromatography (TLC) [17, 18], high-performance liquid chromatography (HPLC) method with UV [18–24], coulometric [25] or MS detection [26, 27]. Pharmacopoeias do not provide any monograph for LXM. But these assay methodologies often suffer from disadvantages of low sensitivity, time-consuming procedures, poor reproducibility and complicated pretreatment or expensive apparatus. FI-CL method is known to be a powerful analytical technique that promises low detection limit, excellent sensitivity, wide linear dynamic range and
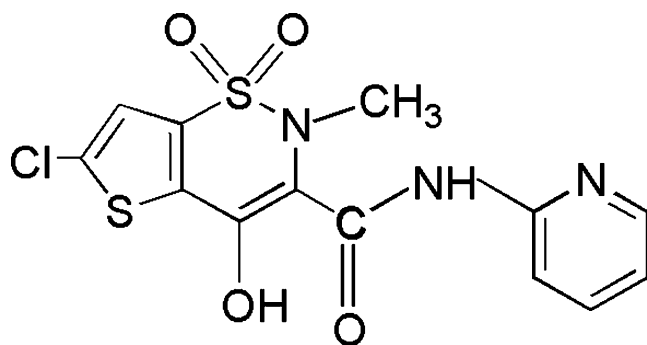
Fig. 1 The structure of lornoxicam

requires relatively simple and inexpensive instrumentation, and has been applied for biomedical, pharmaceutical, and clinical analysis [28, 29]. To the best of our knowledge, however, nothing exists in the literature concerning the CL assay of LXM. The aim of this work, therefore, was to investigate the use of CL in the detection of LXM in pharmaceutical preparations and biological fluids.

In the present work, a FI method with CL detection is developed for the rapid determination of trace amounts of LXM based on the CL reaction between cerium (IV) and LXM using Rh B as energy transfer agent in a micellar medium. The proposed approach offers a good accuracy and precision and has been satisfactorily applied to the determination of LXM in the commercial preparations and biological fluids.

Experimental

Apparatus

The flow system used in this work is represented schematically in Fig. 2. A peristaltic pump was used to deliver all flow streams, each at a flow rate of 3.5 mL/min.
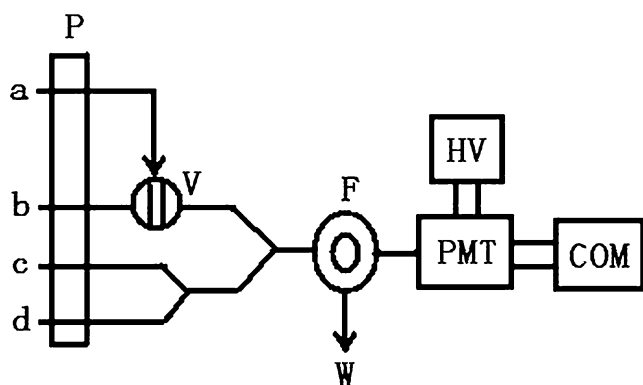

Fig. 2 Schematic diagram of FI-CL analysis system. **a** sample solution; **b** Rh B solution; **c** Ce (IV)+ nitric acid solution; **d** SDBS solution. *P* Peristaltic pump; *V* injection valve; *F* flow cell; *HV* high voltage; *PMT* photomultiplier tube; *W* waste solution; *COM* computer

Polytetrafluoroethylene (PTFE) tubing (0.8 mm i. d.) was used as connection material in the flow system. Injection was operated by means of a six-way valve equipped up with a 100 μL sample loop. The flow cell was a coil of the glass tube that was positioned in front of the detection window of the photomultiplier tuber (PMT). The CL data acquisition and treatment were performed using IFFL-D type flow injection CL data processing system (Xi'an Ruike Electronic equipment Co.). Fluorescence and CL spectra were recorded with F-4500 spectrofluorimeter (Hitachi, Japan).

Chemicals and Reagents

All chemicals were of analytical grade and doubly distilled water was used throughout. LXM and $Ce(SO_4)_2 \cdot 4H_2O$ were purchased from Sigma Chemical Co. (St. Louis, USA). Sodium dodecyl benzene sulfonate (SDBS) and cetyltrimethylammonium bromide (CTAB) were purchased from Signal Aldrich Corporation (Steinheim, Germany). Rh B was from Merck.

The 0.1 mg/mL standard solution of LXM was prepared by dissolving appropriate amount of LXM in 3.0×10^{-3} mol/L sodium hydroxide solution and kept at about 4 °C in a dark bottle. Working standard solutions were prepared by appropriately diluting the stock solution with water before use. The 0.01 mol/L cerium (IV) stock solution was prepared by dissolving the $Ce(SO_4)_2 \cdot 4H_2O$ in 0.7 mol/L HNO_3 solution. The 0.1 mol/L Rh B and SDBS stock solution were freshly prepared by dissolving each reagent in water.

Procedure

In order to obtain good mechanical and thermal stability, the instruments were run for 10 min before the first measurement. As shown in Fig. 2, flow tubes were connected with the LXM standard or sample solution, Rh B solution, Ce (IV) solution and SDBS solution, respectively. LXM standard or sample solution was injected into the carrier stream by a six-way valve quantitatively, which was then merged with acidic Ce (IV) solution and SDBS solution. The mixed solution was delivered to the flow cell, producing CL emission. Calibration graphs were constructed by plotting the relative CL intensity versus the concentration of LXM.

Preparation of Samples

LXM tablets were purchased from local drug stores. No less than 20 tablets were weighed to obtain the average tablet weight and finely powdered. An accurately weighed amount of the powder equivalent to 8.00 mg of LXM was

dissolved with 3 mL of 0.05 mol/L NaOH in a small beaker and transferred into a 50-mL volumetric flask and diluted to the mark with water. The solution was filtered and the residue was washed several times with a 3×10^{-3} mol/L NaOH solution. First 10 mL portion of the filtrate was discarded. An appropriate volume of the filtrate was further diluted appropriately with the same base so that the final LXM concentration was within the linear range of the calibration graph. For injection, the sample was dissolved and diluted with water so that the final concentration was in the working range.

Serum samples were obtained from the local hospital. A known amount of LXM standard solution and 0.2 mL of serum samples were transferred into a centrifuge tube and mixed for 3 min. The potential effect produced by serum proteins and reducing substances was eliminated with 2.0 mL of 0.1 mol/L $Ba(OH)_2$ and 1.8 mL of 0.10 mol/L $ZnSO_4$ according to the reported method [30, 31]. The resultant solution was diluted to 5.0 mL with water and centrifuged at 4000 rpm for 10 min. 1.0 mL of the supernatant clear solution was transferred into a 50.0 mL calibrated flask and diluted to volume with water for analysis. A blank serum sample was set up by treating LXM-free serum in the same way.

Urine samples were collected from the healthy volunteers. No further pretreatment was needed for urine samples except proper dilution in order to make the LXM concentration within the working range. A blank urine sample was set up by treating LXM-free urine in the same way.

Results and Discussion

Optimization of CL Reaction Conditions

A number of the variables affecting the CL reaction were optimized using a univariate approach in order to establish the optimum conditions for the determination of LXM.

The nature and concentration of the acid have very significant influences on this CL reaction because cerium (IV) is readily soluble in acid and becomes stable when dissolved in acid solution. The effects of different acids, such as hydrochloric acid, sulfuric acid, nitric acid, phosphorous acid and acetic acid, on the CL intensity were tested. The highest and most stable emission was obtained from HNO_3-treated cerium (IV) solution. The concentration of HNO_3 in Ce (IV) solution was subsequently optimized. The effect of HNO_3 concentration in cerium (IV) solution was examined in the range of 0.03 to 3.0 mol/L, and the experimental results are shown in Fig. S1. The strongest CL emission occurred while 0.7 mol/L HNO_3 was used to dissolve cerium (IV). Lower or higher concentration of

nitric acid caused a decrease in CL intensity. Then, a 0.7 mol/L nitric acid was chosen as the acidic medium to dissolve cerium (IV) in the following study.

Cerium (IV) was utilized as the oxidant in this CL system and the concentration of it affected both the sensitivity and the linear range of the assay. In order to investigate the effect of cerium (IV) concentration on the CL intensity, the concentration of Ce (IV) in the range $1.0 \times 10^{-5}-1.0 \times 10^{-3}$ mol/L in 0.7 mol/L HNO_3 solution was checked (Fig. S2). The experimental results showed that the maximum CL signal was observed at 3.0×10^{-4} mol/L. Higher cerium (IV) concentration resulted in a decrease of the emission intensity, which may be explained the excess of cerium (IV) might absorb a significant amount of the emitted light [32]. Therefore, 3.0×10^{-4} mol/L cerium (IV) was selected for subsequent experiment.

In an attempt to further improve the sensitivity of the CL intensity, a few of enhancers such as fluorescein, eosin, quinine, acridine, Rh B and Rh 6 G, were chosen and their sensitizing effects on the present CL reaction were tested. The highest CL emission intensity and maximum ratio of signal/noise were obtained only when Rh B was used in the CL system. The effect of Rh B concentration on the CL intensity was also investigated in the range from 1.0×10^{-3} to 1.0 mol/L(Fig. S3). The CL intensity was found to increase with the increasing concentration of Rh B and reached a maximum value at 5.0×10^{-3} mol/L, above which the CL intensity decreased sharply probably because of self-absorption of the emission by Rh B. The reproducibility of the CL signal was unacceptable. Hence, 5.0×10^{-3} mol/L Rh B was chosen as the optimum concentration.

Surfactants are often played a vital role to boost up the emission intensity of CL reaction. Four types of surfactants, SDBS, β-CD, CTAB and TX-100 were used to examine the effect of the sensitization behavior on the CL system. Results showed that the greatest CL signal was appeared by the use of SDBS as the micellar medium. Therefore, SDBS was selected for this work. In this sensitized CL system, the key intermediate may be cationic Ce^{3+} or cationic complex $Ce(LXM)_2^{3+}$. A large number of Ce^{3+} and Ce^{4+} ions were concentrated in the stern layer of the SDBS micelles, while the LXM can easily reach the stern layer of the micelles. So the interaction between Ce^{4+} and LXM might take place, which facilitate the energy transfer between the Ce^{3+} radical ions and LXM. Moreover, the microenvironment created by micelles can protect the excited species from the collisional quenching of light emission, increase the excited state lifetimes and decrease the rate of radiationless energy transfer processes. The stability and reproducibility of the CL system were also greatly improved in SDBS micelles. The effect of SDBS concentration on the relative CL intensity was also investigated in the range of 5.0×10^{-3} to 0.1 mol/L. As illustrated in Fig. S4, maximum CL intensity

Table 1 Figures of merit for determination of LXM

Method	Linear range	Detection limit	Matrix	Ref.
SMC-HPLC-UV	25–1000 ng/mL	6.4 ng/mL	Human blood	[23]
HPLC-UV	0.5–20.0 μg/mL	0.01 μg/mL	Tablets	[18]
	10–200 μg/mL	0.12 μg/mL	Tablets	[19]
RP-HPLC-PDA	0.2–80 μg/mL	0.008 μg/mL	Tablets	[24]
RP-HPLC	0.5–20 μg/mL	0.013 μg/mL	Tablets	[22]
	0.08–20 μg/mL	0.007 μg/mL	Tablets	[21]
HPLC-ESI-MS	0.50–500 ng/mL	0.50 ng/mL	Human plasma	[26]
HPLC-MS	0–1600 μg/L	2.0 μg/L	Human plasma	[27]
HPLC-ED	5–1000 ng/mL	5 ng/mL	Human plasma	[25]
TLC	100–600 ng/spot	80.0 ng/spot	Tablets	[17]
	0.25–6 μg/spot	0.08 μg/spot	Tablets	[18]
Spectrofluorimetry	5–1000 ng/mL		Tablets	[10]
Voltammetry	25–1025 ng/mL	5.4 ng/mL	Human serum	[15]
UV–vis	10–20 μg/mL	0.0789 μg/mL	Tablets	[13]
	1.5–5.5 μg/mL	0.0403 μg/mL	Tablets	[13]
ZOD UV	0.5–35 μg/mL	0.13 μg/mL	Pharmaceutical preparations	[12]
FOD UV	0.2–75 μg/mL	0.06 μg/mL	Pharmaceutical preparations	[12]

SMC semi-micro column; *ZOD* zero order derivative; *FOD* first order derivative; *ED* electrochemical detection

was obtained at a concentration of 9.0×10^{-2} mol/L SDBS, namely under or above 9.0×10^{-2} mol/L, a decrease of the CL intensity was caused. Hence, 9.0×10^{-2} mol/L SDBS was used for the whole experiment.

The flow rate is an important parameter in CL detection because the time taken to transfer the excited product into the flow cell is critical for maximum collection of the emitted light. Too low or too high flow rates result in a decrease or even absence of CL in the flow cell. The optimum flow rate was found to be 3.5 mL/min in each line, and this was used for all further work. Increasing the flow rate above 3.5 mL/min did not significantly enhance the CL intensity and would increase the pressure in the tubes and the consumption of reagents.

Performance of the System for LXM Measurement

Under the selected experimental conditions given above and using the flow-injection system described Fig. 2, the calibration graph of the responses of CL intensity (ΔI) versus the concentration of LXM (c) was linear in the range of 1.0×10^{-10} to 7.3×10^{-8} g/mL, and the detection limit was

4.9×10^{-11} g/mL, which was calculated as the amount of LXM required to yield a net peak three times the standard deviation of the background signal (3σ). The regression equation was $\Delta I = 5.49 \times 10^{10} C + 713$ (C being the LXM concentration, g/mL) with a correlation coefficient of 0.9937 ($n=11$). The relative standard deviation (RSD) was 1.9% for the 3.0×10^{-9} g/mL LXM solution considering to 11 replicate measurements. Figures of merit of comparable methods for determination of LXM were shown in Table 1.

Interference Studies

In order to assess the proposed CL method for the analysis of LXM in pharmaceutical dosage forms and biological samples, the interference of commonly used excipients and additives, co-existing ions and the other compounds on the determination of 3.0×10^{-9} g/mL LXM was investigated. The tolerable concentration ratios of foreign species to 3.0×10^{-9} g/mL LXM at the 5% level were over 1000-fold K^+, Mg^{2+}, NH_4^+, Cl^-, SO_4^{2-}, lactose, galactose, dextrin; 500-fold Ba^{2+}, Pb^{2+}, NO_3^-, CO_3^{2-}, urea, arabic gum, starch; 200-fold Ca^{2+}, Ni^{2+}, EDTA, citrate, saccharose; 100–fold Mg^{2+},

Table 2 Determination results of LXM in pharmaceutical formulations

Sample	Labeled (mg)	Proposed method Found±RSD(%)(n=9)	UV–vis method [13] Found±RSD (%)(n=5)	Added (mg)	Found (mg)	Recovery (%)
tablet 1	8	8.1±2.3	8.06±0.13	5.00	13.2	101.5
tablet 2	8	7.8±1.9	7.9±0.09	10.0	17.9	99.44
Injection1	8	7.9±1.8	7.8±0.2	15.0	23.1	100.4
Injection2	8	8.2±1.9	8.1±0.17	30.0	37.7	99.21

RSD Relative standard deviation

Table 3 Determination of LXM in human serum and urine samples

Sample	Added[a]	Recovery (%) (RSD%, $n=7$)	
	$(10^{-6}$ g/mL)	Serum	Urine
1	0.3	99.3±1.6	94.7±2.2
2	0.6	101±1.9	100±2.1
3	1.0	96.7±2.3	101±0.9
4	3.0	99.1±1.5	103±1.3
5	7.0	102±2.3	97.7±1.8

[a] No dilution

Fig. 4 Fluorescence spectra. **a** Ce (III) (in 0.7 mol/L HNO₃), 3.0× 10^{-4} mol/L, λem=365 nm, λex=290 nm; **b** LXM, 5.0×10^{-7} mol/L, λem=447 nm, λex=290 nm; **c** LXM, 5.0×10^{-7} mol/L, + Ce (IV) (in 0.7 mol/L HNO₃), 3.0×10^{-4} mol/L; λem=367 nm, λex=290 nm; **d** LXM, 5.0×10^{-7} mol/L, + Ce (IV) (in 0.7 mol/L HNO₃), 3.0×10^{-4} mol/L+SDBS, 9.0×10^{-2} mol/L, λem=367 nm, λex=290 nm

Zn²⁺, Al³⁺, Cu²⁺, HPO₄²⁻, mannitol, β-cyclodextrin; 50-fold Co²⁺, HCO₃⁻, H₂PO₄⁻, glucose, sucrose; 20-fold S²⁻, Mn²⁺, L-lysine, ethyl cellulose; 5-fold Fe³⁺; 2-fold C₂O₄²⁻, ascorbic acid.

Application of the Method

Following the procedure detailed in the experimental section, the proposed FI-CL method was applied to the determination of LXM in two commercial pharmaceutical formulations. The results were summarized in Table 2. The *t*-test assumed that there is no significant difference between the results obtained by the proposed method and those obtained by the UV–vis method at the confidence level of 95%.

The serum levels of LXM after a normal therapeutic dose were reported to be in the approximate range of a few tens to several hundreds ng per mL serum [25]. The sensitivity and detection limit achieved by the proposed FI-
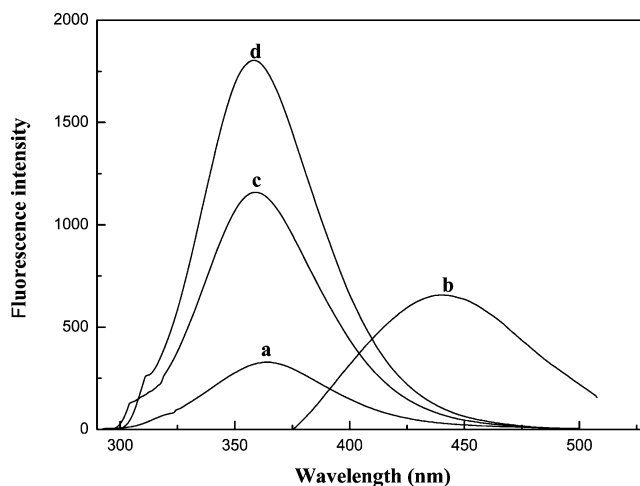
CL technique allows the determination of LXM in biological fluids. To adjust the sample concentration of the drug to within the linear range of determination, after deproteinization and centrifugation of a serum sample the supernatant was used to investigate recovery. The standard addition method was used to avoid matrix effects. The urine samples were diluted appropriately with water and analyzed by the standard addition method. The experimental results were summarized in Table 3. Recovery was from 96.7 to 102% for human serum and from 94.7 to 103% for urine, respectively.
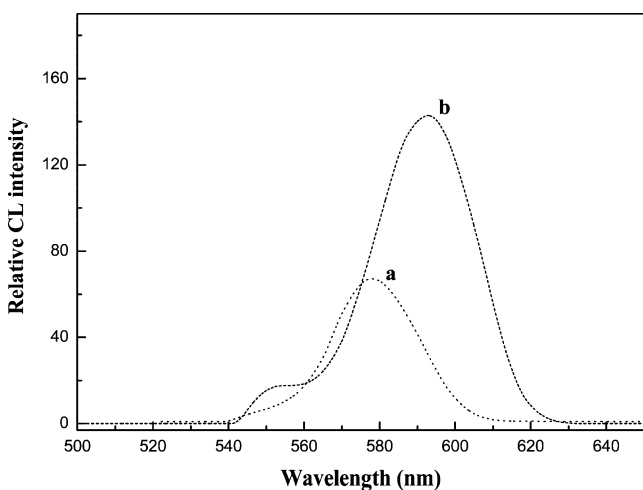

Fig. 3 The CL spectra of Ce (IV)-LXM-Rh B (**a**) and Ce (IV)-LXM-Rh B-SDBS (**b**) system. Concentration: LXM, 7.0×10^{-9} g/mL; Ce (IV) (in 0.7 mol/L HNO₃), 3.0×10^{-4} mol/L; Rh B, 5.0×10^{-3} mol/L; SDBS, 9.0×10^{-2} mol/L
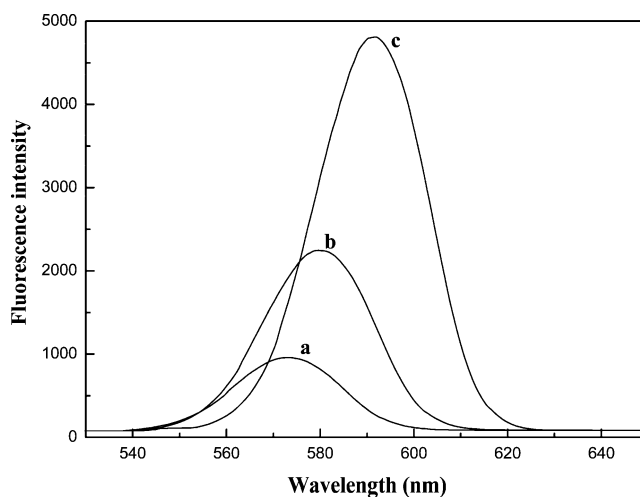

Fig. 5 Fluorescence spectra of Ce (IV)-LXM-Rh B-SDBS system. **a** Rh B, 3.0×10^{-2} mmol/L (without SDBS); **b** Rh B, 7.0×10^{-2} mmol/L; **c** Rh B, 0.1 mmol/L; LXM, 7.0×10^{-9} g/mL; Ce (IV) (in 0.7 mol/L HNO₃), 3.0×10^{-4} mol/L; SDBS, 9.0×10^{-2} mol/L
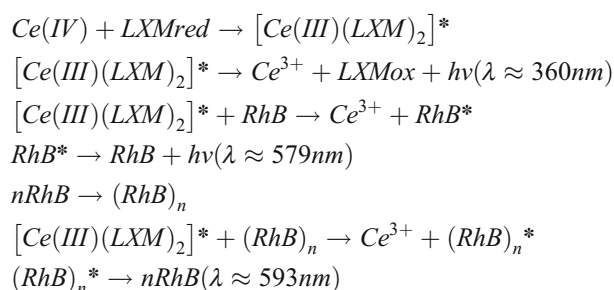
Possible CL Mechanism

In order to propose a possible mechanism of processes taking place in the system studied, a series of specific experiments was carried out.

The CL spectrum of the reaction was examined and the results are shown in Fig. 3. The CL emission peak Ce (IV)-LXM-Rh B is at about 577 nm. But, surprisingly, the CL emission recorded, when LXM and Rh B were reacted with Ce (IV)-SDBS, was at 593 nm which did not coincide with the fluorescence emission spectrum of Rh B (575 nm).

In order to clarify the reasons of the discordance, the fluorescence spectra were investigated. Firstly, the fluorescence spectra of Ce (IV), Ce (III), LXM and the mixture of Ce (IV)-LXM were recorded under the same excite wavelength by means of FL-4500 spectrofluorimeter, respectively and shown in Fig. 4. It was observed that fluorescence spectra for cerium (III) and LXM were obtained at 365 nm and 447 nm respectively. Ce (IV) is non-fluorescence [33]. The spectrum taken from the reaction mixture of Ce(IV)-LXM in a nitric acid medium had a maximum emission wavelength at 330–430 nm, which was very similar to that of Ce (III), and dose not show significant fluorescence emission between the wavelength range 400–500 nm, while the LXM solution gives obvious fluorescent emission in this range. Thus, the Ce (III) ions or the complex formed between Ce (III) and LXM was suggested as the emitter, not to the LXM alone. It is known that in a sensitized CL system the key intermediate frequently involves as a high energetic species in the CL reaction. The $[Ce(C_{13}H_{10}ClN_3O_4S_2)_2]^{3+*}$ complex is most probably such a high energetic intermediate complex in this CL system [34]. Secondly, the absorbance spectra of 3.0×10^{-5} g/mL LXM and the mixture of 3.0×10^{-5} g/mL LXM with 1.0×10^{-4} mol/mL Ce (III) were taken on a UV-2401 spectrophotometer (Shimadzu, Japan). In the presence of Ce (III) the absorbance of LXM at 253 and 374 nm obviously increased and a slight red shift was observed (253 to 257 nm, 374 to 379 nm), which indicated a complex forming between LXM and Ce (III). Molar-ratio method and Job's method [35, 36] were applied in order to examine the stoichiometry of the reaction between LXM and Ce (III), the molar ratio of LXM to Ce (III) is 2:1. Finally, the fluoresce spectra of different concentration of Rh B, 3.0×10^{-2}, 7.0×10^{-2} and 0.1 mmol/L, in the absence and presence of SDBS were explored, as shown in Fig. 5. The emission spectra of Rh B shifted from 575 nm to 593 nm in the presence of SDBS, while those without SDBS almost had no shift. Due to the existence of Rh B$^+$ in the aqueous solution, the sensitized Rh B$^+$ should be more easily aggregated around the micelle of anionic surfactant SDBS. Because of electrostatic effect between anionic surfactant SDBS and Rh B molecules, polymeric Rh B $(RhB)_n$ molecules were formed and excited more easily compared to monomer of Rh B, which led to higher efficiency of energy transfer from the high energetic intermediate complex $[Ce(C_{13}H_{10}ClN_3O_4S_2)_2]^{3+*}$ to $(RhB)_n$ and red shift of the spectrum [37].

Based on the above experimental results, the possible mechanism of the CL reaction was suggested as follows:

$$Ce(IV) + LXMred \rightarrow [Ce(III)(LXM)_2]^*$$
$$[Ce(III)(LXM)_2]^* \rightarrow Ce^{3+} + LXMox + hv(\lambda \approx 360nm)$$
$$[Ce(III)(LXM)_2]^* + RhB \rightarrow Ce^{3+} + RhB^*$$
$$RhB^* \rightarrow RhB + hv(\lambda \approx 579nm)$$
$$nRhB \rightarrow (RhB)_n$$
$$[Ce(III)(LXM)_2]^* + (RhB)_n \rightarrow Ce^{3+} + (RhB)_n^*$$
$$(RhB)_n^* \rightarrow nRhB(\lambda \approx 593nm)$$

Where LXM is the Lornoxicam, Red is the reduced form of LXM; Ox is the oxidized form of LXM, hv is CL emission.

Conclusion

A rapid and economic FI-CL technique for the determination of LXM in SDBS micelles was established for the first time. Compared with the other methods, the present work had a good sensitivity, selectivity, precision and wide linear range, which allowed potential application in pharmaceutical and biological sample analysis. The possible synergetic mechanism of the CL reaction is briefly discussed.

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