Europium sensitized chemiluminescence
determination of rufloxacin

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Abstract

A novel chemiluminescence (CL) flow system for rufloxacin (RFX) combined flow-injection analysis is presented in this paper. It is based on the energy transfer from RFX to europium(III), the intense luminescence instead of the weak CL produced by cerium(IV)–sulfurous acid–RFX CL system can be observed when Eu(III) was added to the system. A narrow and highly intense emission band at 617 nm arose from the excited-state Eu(III) was obtained. Under the optimum experimental conditions, the linear range was 2.0 × 10⁻⁸ to 5.0 × 10⁻⁷ M and the detection limit was 5.0 × 10⁻⁹ M. The method has been successfully applied to determination RFX in dosage form, serum samples and urine samples. The recoveries were 95.0–104.9% for serum and urine samples. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Chemiluminescence; Rufloxacin; Flow-injection analysis; Europium; Energy transfer

1. Introduction

Rufloxacin (RFX), 9-fluoro-2,3-dihydro-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzothiazine-6-carboxylic acid is one of the synthetic antibacterial fluoroquinolone agents of the third generation with antibacterial activity against infections of the urinary and respiratory tract [1]. Therefore, it is of great importance to determine its contents in various biological fluids (blood and serum) and tissues.

Up to now the commonly employed techniques for its pharmacokinetic study, for example, HPLC [2], UV-spectroscopy [3], volumetry [4] and fluorimetry [5] have been used. Although, these methods are relatively sensitive, they suffer from the disadvantages common to the use of complicated equipment and high cost.

Analytical method applying chemiluminescence (CL) coupled with flow-injection analysis (FIA) shows the advantages of simplicity and rapidity, and has been used for the analysis of pharmaceutical compounds [6,7]. However, chemical reactions that result in significant CL emission are few. The lanthanide ions, terbium(III) and europium(III), are unusual in their ability to undergo an energy transfer with certain excited-state organic ligands. The energy transfer from the triplet-state of the ligand to the Eu(III) ion, and the efficiency depend on the match of ligand and lanthanide energy levels [8,9]. Moreover, the lanthanide chelates have characteristics of large Stokes shift and emit narrow emission band, so it is widely applied in liquid chromatography with luminescence detection and in sensitized fluorimetry [10–14]. The
combination of advantages of the CL method with the use of lanthanide ions as luminescence sensitizer provides information on the spectra and structural properties of organic ligands coordinated with lanthanide ions. The energy-transfer properties of the lanthanide ions on CL reactions have been investigated by Kazakov et al. [15]. In our study, a weak CL produced by Ce(IV)-H2SO3-RFX reaction was observed. It is interesting to find that in the presence of Eu(III) it showed a strong enhancement effect on this weak CL. To our knowledge, this is the first time to report Eu(III) sensitized chemiluminescence determination of RFX based on the CL reaction of Ce(IV)-H2SO3. This paper describes detailed observations and determination of RFX in biological fluids and pharmaceutical preparations.

2. Experimental

2.1. Apparatus

The CL–FIA system used for the determination of RFX is schematically shown in Fig. 1. PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow system. RFX was injected into the carrier stream by a six-way injection valve. The CL signal was measured with a photomultiplier tube (operated at −950 V) of a GD-1 weak luminescence analyzer (Xi’an RuiKe Electronic Equipments Company, China). Kinetic characteristics and spectra of the CL system were performed on a BPCL ultra chemiluminescence analyzer (Institute of Biophysics, Academia Sinica, China). Fluorescence spectra were recorded with a Hitachi-850 spectrofluorimeter (Japan) equipped with a 150 W xenon lamp. Absorption spectra were recorded on a Shimadzu-UV 250 spectrophotometer (Japan).

2.2. Reagents

All chemicals were of analytical reagent grade; distilled and deionized water was used throughout. Stock standard solution (1.0 × 10⁻³ M) of RFX (Institute of Medical Biotechnology, Beijing, China) was prepared by dissolving RFX in water, working solutions were freshly prepared by dilution of stock solution with water. Europium chloride stock solution (0.1 M) was prepared by dissolving 1.76 g Eu₂O₃ in 5.0 ml HCl (11.6 M) at 95°C and evaporating the solution to be almost dry before diluting to the 100 ml with water, stored in a plastic bottle. Cerium(IV) sulfate (2.0 × 10⁻³ M) in 0.01 M sulfuric acid and sulfurous acid were prepared daily.

2.3. Procedures for determination of RFX

RFX standard solution was injected into the flow system through the six-way injection valve and mixed with H₂SO₃, Ce(IV), and Eu(III) reagents; transferred into the CL cell, then gave rise to an evident CL signal immediately. The relative CL intensity /ΔI (the difference of CL intensity between RFX standard solution and the reagent blank without RFX) was proportional to concentration of RFX. A calibration graph of relative CL intensity versus the RFX concentration was prepared for the determination of the RFX content of the samples.

2.4. Procedures for determination of RFX in a pharmaceutical preparation

The contents of five capsules were weighed to obtain the mean mass per capsule. An accurately weighed sample of the homogenized capsule powder was
dissolved with water in a small conical flask. The solution was filtered and the residue was washed with water several times, then diluted appropriately with water in a calibrated flask. A suitable aliquot was analyzed by the CL–FIA procedure.

2.5. Procedures for determination of RFX in human serum

About 0.2 ml serum sample was deproteinized by adding 2.0 ml of 0.1 M Ba(OH)₂ and 1.8 ml of 0.1 M ZnSO₄ in a 6 ml plastic centrifuge tube, which was then centrifuged for 5 min at 10,000 rev/min. The centrifugate was diluted with water so as to obtain a concentration of RFX in the range of linearity previously determined. The recovery of serum samples containing RFX was determined by the standard addition method.

3. Results and discussion

3.1. Optimization of experimental variables

The effect of sulfuric acid concentration in Ce(IV) solution on CL was studied. The CL emission was the highest at 2.0 × 10⁻⁴ M Ce(IV) containing 0.01 M sulfuric acid, above which it decreased. At concentrations lower than 0.01 M sulfuric acid, the remarkable decrease in CL intensity is due to hydrolysis of Ce(IV) forming cerium hydroxide. Therefore, 0.01 M sulfuric acid was used for subsequent work.

The effect of Ce(IV) concentration in the range of 0 to 6.0 × 10⁻⁴ M on the CL intensity was also examined. As the concentration of Ce(IV) increased up to 2.0 × 10⁻⁴ to 3.0 × 10⁻⁴ M, maximum and constant CL emission intensity was observed. Above 3.0 × 10⁻⁴ M of Ce(IV) concentration, the emission intensity was decreased. Hence, a concentration of 2.0 × 10⁻⁴ M of ceric salt was used for further work.

The maximum CL intensity was observed as the concentration of H₂SO₃ was 0.5% (v/v), and then decreased rapidly with increase of the H₂SO₃ concentration. Above 3.0 × 10⁻⁴ M of Ce(IV) concentration, the emission intensity was decreased. Hence, a concentration of 2.0 × 10⁻⁴ M of ceric salt was used for further work.

The maximum CL intensity was observed as the concentration of H₂SO₃ was 0.5% (v/v), and then decreased rapidly with increase of the H₂SO₃ concentration. Therefore, 0.5% (v/v) H₂SO₃ was chosen for the present work.

Some compounds and ions, such as rhodamine 6G, eosine, riboflavin, rhodamine B, quinine sulfate, fluorescein, 8-hydroxyquinoline, Eu(III), and La(III) were investigated for enhancing the weak signal of CL reaction of Ce(IV)–H₂SO₃–RFX. We found that Eu(III) offers the highest CL intensity as the energy for chemical excitation produced during the Ce(IV)–H₂SO₃–RFX reaction matches the energy requirement for Eu(III) excitation. A possible mechanism is discussed later. With increasing concentration of Eu(III), the relative CL intensity first increases, but then decreases at Eu(III) concentration over 5.0 × 10⁻⁴ M. Hence, a 5.0 × 10⁻⁴ M Eu(III) concentration was chosen for further study.

3.2. The kinetic characteristics of the CL reaction

The kinetic characteristics of the CL reaction were examined as shown in Fig. 2. It can be seen that the reaction was so rapid that the CL intensity reached to maximum at 1 s after the reaction was started, then attenuated quickly to baseline in 15 s.

3.3. Determination of studied drug

The calibration graph for the determination of RFX was conducted under the optimal conditions. Excellent linearity ∆I = 15.08 × 10⁸C – 20.65 (where C represents the concentration of RFX (M)) with a linear correlation coefficient (r) of 0.9993 was obtained in the range 2.0 × 10⁻⁸ to 5.0 × 10⁻⁷ M RFX. The relative standard deviation was 1.5% for 11 determinations of 1.0 × 10⁻⁷ M RFX. The detection limit for RFX was calculated from the standard deviation of the blank (the reagent blank without RFX, n = 20) (3σ) as 5.0 × 10⁻⁹ M RFX.
Table 1
Tolerance to different substances in the determination of RFX

<table>
<thead>
<tr>
<th>Species added</th>
<th>Maximum tolerable mole ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺, Na⁺, Mg²⁺, Ca²⁺, Ni²⁺, Cu²⁺, SO₄²⁻, NO₃⁻, Cl⁻</td>
<td>1000</td>
</tr>
<tr>
<td>Co²⁺, Pb²⁺</td>
<td>500</td>
</tr>
<tr>
<td>Zn²⁺, Al³⁺, starch, glucose, dextrin</td>
<td>100</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>50</td>
</tr>
<tr>
<td>I⁻</td>
<td>5</td>
</tr>
</tbody>
</table>

3.4. Study of interferences
An extensive interference study was carried out with a view to determining RFX in real samples. Samples containing 1.0 × 10⁻⁷ M RFX and various concentration of the foreign substance were injected into the flow system. A substance was considered as no interference if the variation of the CL peak height was <±10%. Most anions and cations have no interference because the tolerated ratios were in most cases far higher than those at which they are normally encountered in real samples. Dextrin and starch exist in the pharmaceutical preparations, and they can be eliminated by filtration in order to determine RFX concentration. The results obtained are summarized in Table 1.

Some organic species, such as Vitamins B₁, B₂, serum albumin and myoglobin seriously interfere the CL signals. In the urine samples, the concentrations of these species are much lower than those in the serum samples. Dilution could be considered to minimize the interference in the application of urine samples, while in the serum samples, ZnSO₄ and Ba(OH)₂ should be added to precipitate the interferent before the determination.

Table 2
Results for the determination of RFX in capsules (n = 5)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount (mg)</th>
<th>Added (× 10⁻⁸ M)</th>
<th>Found (× 10⁻⁸ M)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Labeled (mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>± S.D. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsule 1</td>
<td>100</td>
<td>4.0</td>
<td>3.89</td>
<td>97.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>6.04</td>
<td>100.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0</td>
<td>9.95</td>
<td>99.0</td>
</tr>
<tr>
<td>Capsule 2</td>
<td>100</td>
<td>4.0</td>
<td>3.86</td>
<td>96.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>6.05</td>
<td>100.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0</td>
<td>9.88</td>
<td>98.5</td>
</tr>
</tbody>
</table>

3.5. Analytical applications
The proposed method was applied to the determination of RFX in two pharmaceutical preparations. The results obtained and the labeled contents are given in Table 2. There were no significant differences between labeled contents and those obtained by the proposed method. Recovery studies were also performed on each of the analyzed samples by recommended treatment. Recoveries ranged from 96.5 to 100.8%.

The information on the single-dose pharmacokinetics of RFX [16] indicates that the maximum serum level following a 400 mg oral dose is 3–6 μg ml⁻¹, i.e. 7.5 × 10⁻⁶ to 1.5 × 10⁻⁵ M. In long term treatment 200 mg oral doses of RFX, preceded by a loading dose of 400 mg, are needed to establish a urinary concentration of 29.75 μg ml⁻¹, i.e. 7.4 × 10⁻⁵ M, between 48 and 72 h after the final administration [17]. A healthy volunteer administered 100 mg RFX capsule, then after 12 h real urine samples were investigated. In order to make the sample concentrations of the drug within the linear range of determination, urine and serum samples were diluted 500- and 220-fold, respectively, and the recoveries of urine and serum samples containing RFX were determined by the standard addition method [18]. The results obtained are listed in Tables 3 and 4.
Table 4
Results for the determination of RFX in serum samples (n = 5)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (× 10^{-7} M)</th>
<th>Found (× 10^{-7} M)</th>
<th>Mean recovery (%)</th>
<th>R.S.D (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 1</td>
<td>1.0</td>
<td>1.005</td>
<td>100.5</td>
<td>4.8</td>
</tr>
<tr>
<td>Serum 2</td>
<td>2.0</td>
<td>2.099</td>
<td>104.9</td>
<td>1.6</td>
</tr>
</tbody>
</table>

3.6. Formation of Eu(RFX)_{3+}

In order to investigate the possible mechanism of Eu(III)-RFX-Ce(IV)-H_{2}SO_{3} CL system, the absorption and the fluorescence excitation and the emission spectra of RFX and Eu(III)-RFX were recorded, respectively. From the absorption spectra of RFX and Eu(III)-RFX, it can been seen that there are three absorption bands for each of them, but the absorption peak position of Eu(III)-RFX has a slight shift, and the absorbance of Eu(III)-RFX increases, which implies a Eu(III)-RFX complex has formed. Fig. 3 shows the excitation and the emission spectra of RFX and Eu(III)-RFX systems. The emission spectra show that the native fluorescence emission wavelength of RFX is 510 nm (Fig. 3a). The results also prove the formation of the Eu(III)-RFX complex since the free Eu(III) aqueous solution under the same conditions fluoresces too weakly to be observed. The energy absorbed by RFX is transferred efficiently to Eu(III) by an intramolecular energy-transfer process, Eu(III) is then excited and subsequently emits intensely.

Fig. 3. Fluorescence excitation (a, b) and emission spectra (a’, b’) of RFX and Eu(III)-RFX in a): λ_{ex} = 510 nm, λ_{em} = 580 nm, c_{RFX} = 5.0 × 10^{-6} M, c_{Eu^{3+}} = 8 × 10^{-3} M; in b): λ_{em} = 617 nm, λ_{ex} = 350 nm, c_{RFX} = 5.0 × 10^{-6} M, c_{Eu^{3+}} = 8 × 10^{-3} M.

We used the molar ratio method and Job’s method of continuous variation [19,20] to examine the composition of the Eu(III)-RFX. As shown in Fig. 4, the molar ratio of RFX to Eu(III) is 2:1.

3.7. Possible mechanism

Excited sulfur dioxide has been considered as the emitting species during the oxidation of sulfite by Ce(IV), which was proposed by Stauff and Jaechke [21] in 1975. The CL reaction may be due to the fact that Ce(IV) can oxidize sulfurous acid to a hydrogen sulfite radical HSO_{3}^{-}, and then two HSO_{3}^{-} radicals combine to produce SO_{2}^{•−}, SO_{2}^{•−} will give the excited intermediate product SO_{2}^{•−} [22,23] which emits radiation in the spectral region 300–450 nm [24].

The CL spectra of Ce(IV)-H_{2}SO_{3}–Eu(III) and Eu(III)-RFX-Ce(IV)-H_{2}SO_{3} are shown in Fig. 5. The CL peaks of the two systems are located at 620 nm. For the Ce(IV)-H_{2}SO_{3}–Eu(III) system, the energy of SO_{2}^{•−} is transferred to Eu(III). But for Eu(III)-RFX-Ce(IV)-H_{2}SO_{3} system, the energy of SO_{2}^{•−} is mainly transferred to RFX (in the Eu(III)-RFX complex). According to [11], certain functional groups, notably benzoyl groups or nitrogen heterocycles, have triplet-state energies that match those of the excited states of Eu(III). The excitation of Eu(III) takes place through the intermolecular energy transfer from SO_{2}^{•−} to the triplet-state of the ligand and then an intramolecular energy transfer to Eu(III), followed by 5D_{0} emission of Eu(III) of Eu(RFX)_{3+} complex. However, the process of energy transfer from SO_{2}^{•−} to 5D_{0} of Eu(III) may not be excluded in...
the Eu(III)-RFX-Ce(IV)-H$_2$SO$_3$ CL system. From Fig. 5, we can see that an increase in the luminescence intensity of Eu(III) in Eu(III)-RFX-Ce(IV)-H$_2$SO$_3$ can be observed. This is not only because of removal of inner-sphere coordinated water molecules but also energy transfer from RFX to Eu(III). The mechanism stated above can be expressed as follows:

$$\text{Ce}^{(IV)} + \text{HSO}_3^- \rightarrow \text{Ce}^{(III)} + \text{HSO}_3^\bullet \quad (1)$$

$$2\text{HSO}_3^\bullet \rightarrow \text{S}_2\text{O}_6^{2-} + 2\text{H}^+ \quad (2)$$

$$\text{SO}_2^\bullet + \text{Eu}(\text{RFX})^{2+} \rightarrow \text{SO}_2 + \text{Eu}(\text{RFX})^{3+} \quad (4)$$

$$\text{Eu}(\text{RFX})^{3+} \rightarrow \text{Eu}(\text{RFX})^{2+} + h\nu \quad (6)$$

4. Conclusion

The proposed Eu(III)-RFX-Ce(IV)-H$_2$SO$_3$ CL flow system with Eu(III) as energy acceptor shows an advantage of the intense CL band over the other lanthanide ions and fluorophore. Because of its high sensitivity, selectivity and accuracy, it was successfully used in the determination of RFX in pharmaceutical preparations and biological fluids, especially in the determination of urine samples without any prehandling but only by appropriate dilution of the samples.

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