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*ORIGINAL RESEARCH*

# Flow-injection with enhanced chemiluminescence detection of ofloxacin in human plasma

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ABSTRACT: A simple, rapid and sensitive method for the determination of ofloxacin in plasma has been developed based on flow-injection analysis with enhanced chemiluminescence (CL) detection. This method employs the CL reaction of cerium(IV) and sulphite sensitized by a Tb(III)–ofloxacin complex. Plasma samples were deproteinized with acetonitrile before analysis. Under optimal conditions, the method allowed the quantitative analysis of ofloxacin in plasma over the range of 22.5–900 ng/mL with a detection limit of 7.5 ng/mL ($3\sigma$). The intra-day and inter-day precision was < 3.0% ($n = 11$) and < 7.0% ($n = 3$), respectively. The method was a convenient tool, rapid (20 s/analysis) and well suited for pharmacokinetics studies in which thousands of samples must be analysed daily. It was applied to pharmacokinetics studies of ofloxacin. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: human plasma; ofloxacin; flow-injection; chemiluminescence; terbium(III); cerium(IV); sulphite

## INTRODUCTION

Ofloxacin, (±)9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperaziny)-7*H*-oxo-7*H*-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid, is one of the third-generation members of quinolone synthetic antibiotics, with a broad spectrum of activity against Gram-positive and Gram-negative bacteria through inhibition of DNA gyrase (1, 2). It is widely used in therapies against inflammation (3). The drug's effect is concentration-dependent and its antibacterial effect is closely related to its plasma concentration. Therefore, pharmacokinetic studies of ofloxacin are of great importance. Fig. 1 shows the structure of ofloxacin. Current methods used for pharmacokinetic studies of ofloxacin are mainly HPLC with UV or fluorescence detection (4–6). However, the expense of HPLC is high, and the procedures for the preparation of biological samples are laborious, expensive and time-consuming, which makes the development of more simple and effective methods necessary. In recent years, many other detection methods have been developed for ofloxacin, such as HPCE with UV, fluorescence or laser-induced fluorescence detection (7, 8), polarography (9), UV/VIS spectrophotometry (10), fluorimetry (11, 12), flow-injection using UV detection (13), flow-injection coupled with CL detection (14) and electrochemically modified methods (15). Equipment for HPCE and fluorimetry is expensive and the sensitivity of UV or electrochemical modified methods is low, therefore few of these methods have been applied to pharmacokinetics studies.

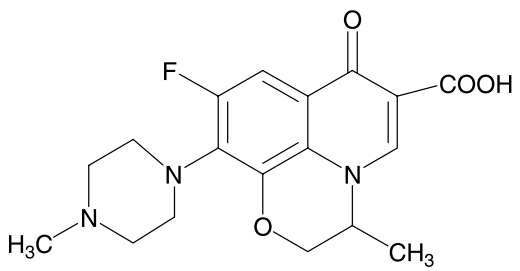


**Figure 1.** Structural scheme of ofloxacin.

Flow-injection coupled with CL detection has great potential in pharmacokinetic studies of many drugs (16–18). Sophisticated apparatus is not necessary in this method, as the energy required for CL emission comes from the chemical reaction, not from an excitation light beam. The procedures for pretreatment are simple, as only deproteinization is required before detection. Furthermore, analysis time is short, each analysis needing only about 20 s, and this is especially important when thousands of samples need to be analysed daily, as in pharmacokinetic studies. Despite these advantages, there are few reports of its application in pharmacokinetic studies, due to the interference of biological fluids with CL signals, especially for plasma and biological tissues. Therefore, it is necessary to develop more selective CL systems and to pre-treat biological samples appropriately. It was reported that Ce(IV)–$Na_2SO_3$ was a highly selective system for the determination of fluoroquinolones (16) but that it lacked sensitivity. It has also been reported that the addition of lanthanide ions, such

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as terbium(III), could increase the CL signal of this system (19, 20). The lanthanide ions form complexes with fluoroquinolones and emit strong emission upon excitation. The complexation reaction was highly selective and the interference from biological samples could be effectively avoided, and plasma concentration of fluoroquinolones could be easily detected after simple pretreatment.

In the present study, the CL reaction of Ce(IV)–$Na_2SO_3$ enhanced by terbium(III) was applied to the flow-injection determination of ofloxacin in human plasma. Compared to HPLC, it is simple, rapid, highly sensitive and precise.

## MATERIALS AND METHODS

### Reagents

Analytical grade reagents and deionized, redistilled water were used throughout. Ofloxacin was purchased from the National Institute for the Control and Biological Products (Beijing, People's Republic of China). $(NH_4)_2Ce(NO_3)_6$ and $Na_2SO_3$ were obtained from Beijing Xinhua Chemical Reagent Factory, and Beijing, the Medical Co. of China, Beijing, respectively. $Tb_4O_7$ was from Beijing Chemical Industry Group, Beijing.

A stock solution of ofloxacin was prepared by dissolving ofloxacin in hydrochloric acid (0.005 mol/L) and was diluted to the required concentration before use. A standard stock solution of Tb(III) was prepared by dissolving 934.5 mg $Tb_4O_7$ in 15.0 mL HCl (11.6 mol/L) at 95°C, evaporating the solution to dryness, then diluting it to 500 mL with water. The cerium(IV) solution was prepared by dissolving $(NH_4)_2Ce(NO_3)_6$ in 0.00011 mol/L HCl solution. $Na_2SO_3$ was dissolved in redistilled water daily.

### Apparatus

The schematic diagram of the flow-injection system is shown in Fig. 2. Two peristaltic pumps (Beijing Haiguang Instrument Co. China) and a six-way injection valve were used to deliver flow streams into this system. All components were connected by PTFE tubes (0.8 mm i.d.). Sample solution was injected into the carrier stream ($Na_2SO_3$) and then was blended with the mixture of Tb(III) and Ce(IV) that then flowed into the quartz flow cell. The flow cell was positioned directly facing the window of the photomultiplier tube. The CL signal in the flow cell was detected and recorded with a computerized ultra-weak luminescence analyser (type BPCL, manufactured at the Institute of Biophysics, Academia Sinica, Beijing, China). Data acquisition and analysis were performed using BPCL software.

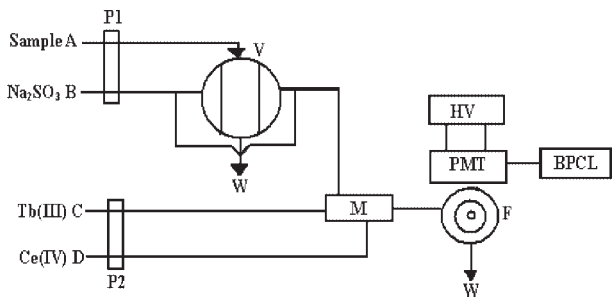


**Figure 2.** Schematic diagram of the CL flow-injection analysis system for ofloxacin determination. P1, P2 peristaltic pump; V, injection valve; F, flow cell; M, mixer; W, waste; HV, high voltage; PMT, photomultiplier tube; BPCL, BPCL workstation.

### Procedures for sample pretreatment

Ofloxacin was transferred with a syringe to the centrifuge tube, 0.5 mL human plasma was added and then mixed, using a vortex agitator for 10 s. Next, 4.0 mL acetonitrile was added to deproteinize the mixture. It was shaken on the vortex agitator for 3 min, then centrifugated for 15 min (3000 r.p.m.). 4.1 mL of the clear supernatant was transferred to another centrifuge tube and diluted to 10.0 mL before analysis.

## RESULTS AND DISCUSSION

### Optimization of experimental conditions

Redox reaction is the basis of CL emission. The emission intensity was greatly affected by oxidants, reductants, sensitizers and acids. Organic solvents used in sample pretreatment also influenced the CL behaviour. Therefore, the effects of deproteinization reagents, oxidants, reductants, sensitizers and acids were investigated, together with the effect of the flow rate.

Pretreatment of biological samples is of great importance in pharmacokinetics studies. With this method, the main step was to remove proteins efficiently. Different deproteinization reagents were examined and the results are shown in Fig. 3. It can be seen that reagents such as $CH_3OH$, $Cl_3CCOOH$ and $ZnSO_4/Ba(OH)_2$ suppressed the CL emission strongly, while the effect of $CH_3CN$ was relatively weak. Therefore, $CH_3CN$ was the most efficient deproteinization reagent and it was employed in the following studies.

The ratio of deproteinization reagent to plasma was further investigated, since it would affect the extraction recovery and the CL emission. The relationship between $CH_3CN$ ratio and CL intensity is shown in Fig. 4. The results indicated that, with increasing ratio of $CH_3CN$, the CL intensity was enhanced continuously as higher extraction recovery was obtained. However, the signal decreased slightly when the ratio of $CH_3CN$ exceeded 8. Therefore, the optimal ratio of plasma:$CH_3CN$ was 1:8.

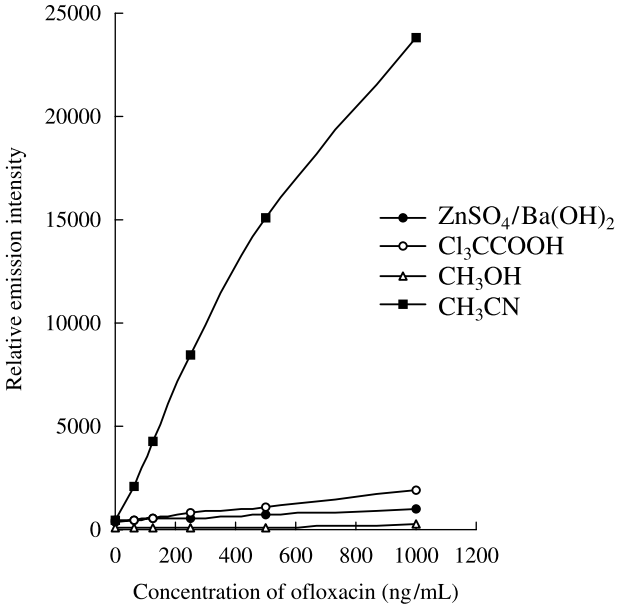


**Figure 3.** Comparison of different deproteinization reagents. $Na_2SO_3$, $7.0 \times 10^{-4}$ mol/L, Ce(IV), $2.0 \times 10^{-4}$ mol/L; Tb(III), $5.0 \times 10^{-4}$ mol/L; HCl, $8.1 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; voltage −800 V.

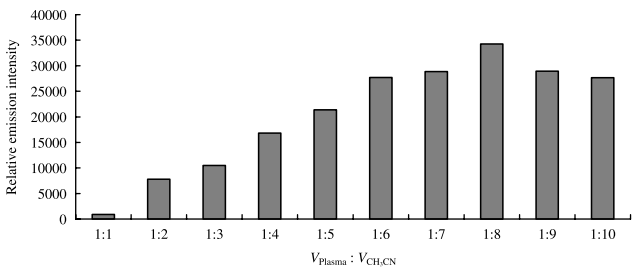


**Figure 4.** Effect of plasma:$CH_3CN$ ratio on CL emission. $Na_2SO_3$, $7.0 \times 10^{-4}$ mol/L; Ce(IV), $2.0 \times 10^{-4}$ mol/L; Tb(III), $5.0 \times 10^{-4}$ mol/L; HCl, $8.1 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; voltage, −800 V; plasma concentration of ofloxacin, 506 ng/mL.

The oxidant in the CL detection reaction can affect detection sensitivity. Therefore, different oxidants, including $(NH_4)_2Ce(NO_3)_6/H^+$, $KMnO_4/H^+$, $H_2O_2$, $NaIO_4/H^+$, $K_2Cr_2O_7/H^+$ and $K_3Fe(CN)_6$, were tested in the concentration range $0.5 \times 10^{-4}$–$7.0 \times 10^{-4}$ mol/L and the relative emission intensity was measured (Fig. 5). The results showed that the signals were quite low with $H_2O_2$, $NaIO_4/H^+$, $K_2Cr_2O_7/H^+$ and $K_3Fe(CN)_6$ as oxidants. The sensitivity was high at certain concentrations using $KMnO_4/H^+$, but the emission intensity decreased sharply with a small deviation from that concentration. In contrast, using $(NH_4)_2Ce(NO_3)_6/H^+$, the CL intensity reached a plateau when the concentration was $> 4.0 \times 10^{-4}$ mol/L, therefore this concentration of Ce(IV) was used in further work.

$(NH_4)_2Ce(NO_3)_6$ is hydrolysed in neutral or basic solution, therefore acids were added to prevent its

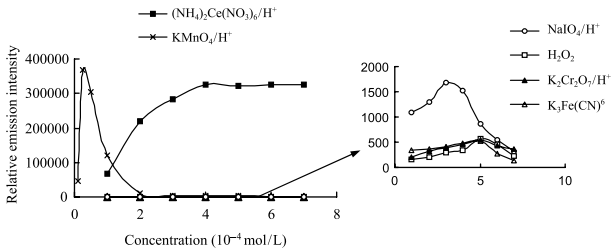


**Figure 5.** Effects of different oxidants on CL emission. $Na_2SO_3$, $7.0 \times 10^{-4}$ mol/L; Tb(III), $8.0 \times 10^{-4}$ mol/L; HCl, $1.1 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; voltage, −800 V; plasma concentration of ofloxacin, 450 ng/mL.

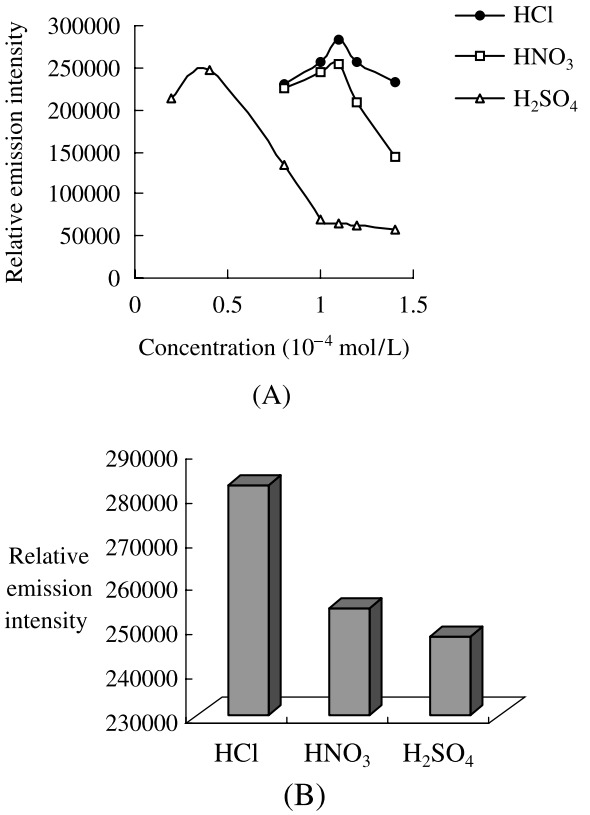


**Figure 6.** Comparison of different acids on CL intensity. $Na_2SO_3$, $7.0 \times 10^{-4}$ mol/L; Tb(III), $8.0 \times 10^{-4}$ mol/L; Ce(IV), $4.0 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; voltage, −800 V; plasma concentration of ofloxacin, 450 ng/mL.

hydrolysis and to increase the CL intensity at the same time. HCl, $H_2SO_4$ and $HNO_3$ were tested and the results are compared in Fig. 6A. The peak emission intensity with different acids is further compared in Fig. 6B. The results showed that the enhancement effect of HCl was more significant compared with $H_2SO_4$ and $HNO_3$ maximum emission intensity could be obtained at a HCl concentration of $1.1 \times 10^{-4}$ mol/L. Therefore, $(NH_4)_2Ce(NO_3)_6$ was dissolved in $1.1 \times 10^{-4}$ mol/L HCl in the present work.

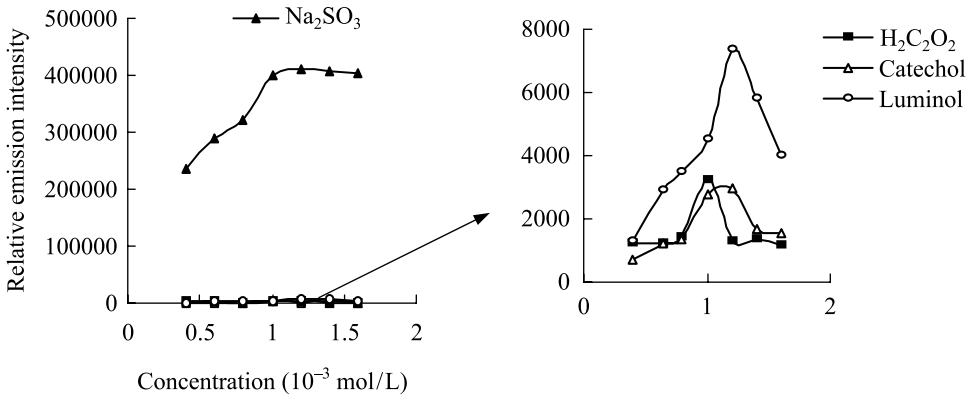


**Figure 7.** Effects of different reductants on CL emission. Ce(IV), $4.0 \times 10^{-4}$ mol/L; Tb(III), $8.0 \times 10^{-4}$ mol/L; HCl, $1.1 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; voltage, −800 V; plasma concentration of ofloxacin, 450 ng/mL.

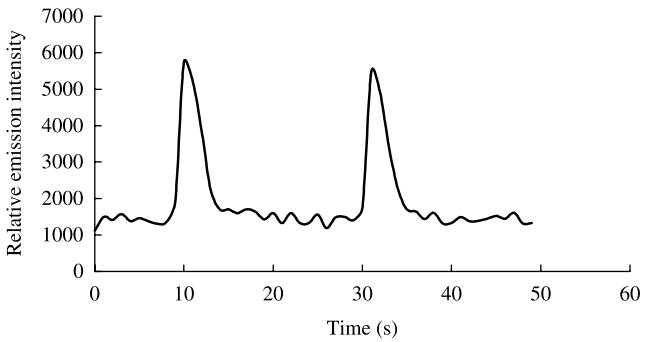


**Figure 8.** The emission intensity without sensitizer. Ce(IV), $4.0 \times 10^{-4}$ mol/L; $Na_2SO_3$, $1.0 \times 10^{-3}$ mol/L; HCl, $1.1 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; voltage, −800 V, plasma concentration of ofloxacin, 450 ng/mL.

The reductant was another important factor in the CL reaction, and the CL efficiency of different reductants varied greatly. Four reductants were compared, including $Na_2SO_3$, $H_2C_2O_2$, luminol and catechol (Fig. 7). It can be clearly seen that $Na_2SO_3$ was the most efficient reductant. Further analysis indicated that the CL intensity increased and then plateaued at a $Na_2SO_3$ concentration of $1.0 \times 10^{-3}$ mol/L. Hence, this concentration was employed in the subsequent work.

As mentioned above, ofloxacin can sensitize the CL reaction of $(NH_4)_2Ce(NO_3)_6$ and $Na_2SO_3$, but the emission was rather weak and was insufficiently sensitive to determine the plasma concentration of ofloxacin. Further experiments showed that the relative emission intensity was less than 6000 for 450 ng/mL of ofloxacin (see Fig. 8). To meet the requirements of *in vivo* analysis, sensitizers should be added to increase the sensitivity. Six sensitizers, including $Tb^{3+}$, $Eu^{3+}$, $La^{3+}$, rhodamine 6G (Rh6G), quinoline (Quin) and fluorescein (Fluo) were investigated in our work and the CL intensity is compared in Fig. 9. From the results, we can clearly see that the sensitizing effect of Tb(III) was much higher than the other compounds tested. Further comparison of peak emission intensity for each sensitizer is also shown in Fig. 10. Based on these results, it could be concluded that Tb(III) was the most efficient sensitizer in this CL system. The optimal concentration of Tb(III) was determined to be $8.0 \times 10^{-4}$ mol/L and this concentration was used in the following experiments.

A further experiment has been performed to prove the sensitizing effect of Tb(III). The same sample was detected by the Ce(IV)–$Na_2SO_3$ and Ce(IV)–$Na_2SO_3$–Tb(III) systems, respectively. The sample was detected in two parallel cycles and the results are shown in Fig. 11. The signal improved nearly 80-fold when Tb(III) was employed as the sensitizer, and hence is a highly sensitive and selective sensitizer for the determination of ofloxacin in plasma.

Besides the above factors, the negative voltage applied to the detector in the BPCL analyser and the flow rate would also affect the CL emission. It could be seen that the CL emission intensity was enhanced sharply with the increase of absolute voltage (Fig. 12). But the noise increased simultaneously and the signal was unstable when the voltage exceeded –1100 V. Therefore, the voltage was fixed to –1100 V in subsequent experiments. To investigate the influence of flow rate, it was ranged from 0.6 mL/min to 2.7 mL/min and the emission intensity was recorded (Fig. 13). The results showed that the signal increased with an increase in peristaltic pump speed. However, high flow rate would require larger amounts of sample, so the flow rate was set at 1.8 mL/min in the present work.

## Mechanism

Based on the above results and the related literature (14), the following mechanism was proposed. Under acidic conditions, $Na_2SO_3$ was oxidized by Ce(IV) and

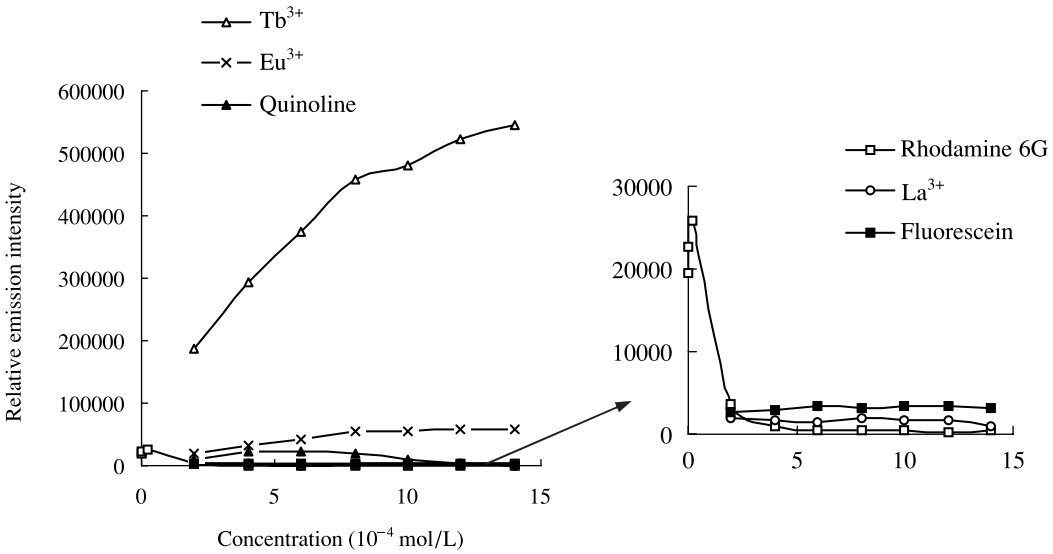


**Figure 9.** Effects of different sensitizers upon the signal intensity. Ce(IV), $4.0 \times 10^{-4}$ mol/L; $Na_2SO_3$, $1.0 \times 10^{-3}$ mol/L; HCl, $1.1 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; voltage, −800 V; plasma concentration of ofloxacin, 450 ng/mL.

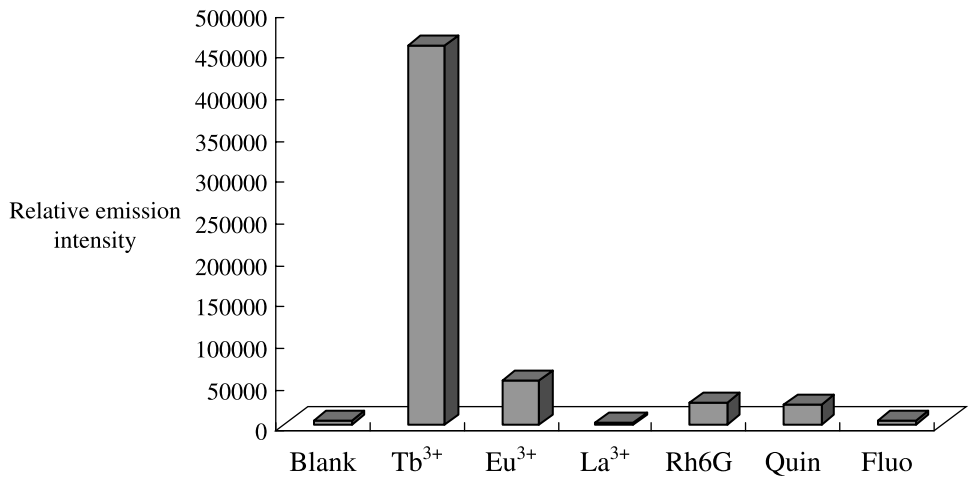


**Figure 10.** Comparison of peak emission intensity with different sensitizers. Ce(IV), $4.0 \times 10^{-4}$ mol/L; $Na_2SO_3$, $1.0 \times 10^{-3}$ mol/L; HCl, $1.1 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; voltage, −800 V; plasma concentration of ofloxacin, 450 ng/mL.

excited sulphur dioxide ($SO_2$*) was produced. $SO_2$* could emit CL, although the emission intensity was very weak due to the low luminescence efficiency of $SO_2$*. By introducing a fluorophore with a maximum absorption in the emission range of $SO_2$* (300–450 nm), the CL intensity could be enhanced (19, 20). In our experiments, Tb(III) was used and it could form a complex with ofloxacin. The energy of $SO_2$* would be transferred to the complex, then a intra-molecule energy transferring process occurred and Tb(III) was excited. When the excited Tb(III) returned to the ground state, fluorescence would be emitted. According to this theory, the energy transfer process is shown as below, where OFLX represents ofloxacin.

$$Ce(IV) + Na_2SO_3 + H^+ \rightarrow SO_2^*$$

$$SO_2^* + Tb(III)\text{–}OFLX \rightarrow SO_2 + Tb(III)\text{–}OFLX^*$$

$$Tb(III)\text{–}OFLX^* \rightarrow {}^*Tb(III)\text{–}OFLX$$

$${}^*Tb(III)\text{–}OFLX \rightarrow Tb(III)\text{–}OFLX + h\nu$$

## Calibration and detection limits

Ofloxacin calibration samples were prepared by adding standard ofloxacin solution to 0.5 mL drug-free human plasma. The calibration data under the optimal conditions are summarized in Table 1. The calibration graph for plasma ofloxacin was linear in the ranges 22.5–225 ng/mL and 225–900 ng/mL. The detection

 

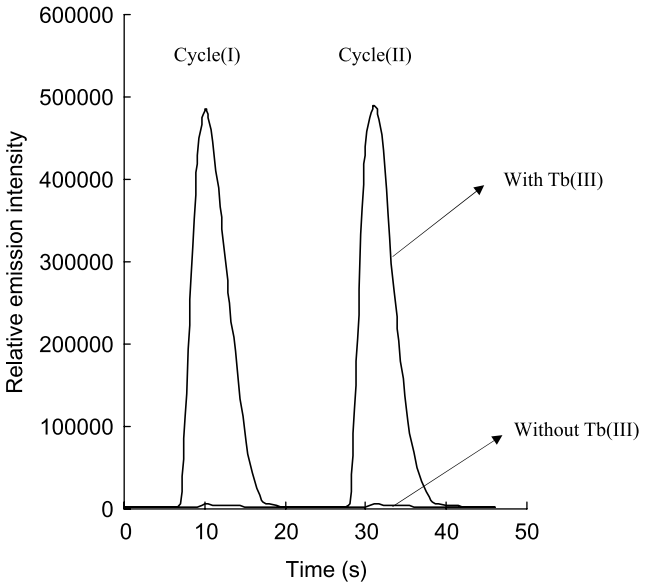


**Figure 11.** The effect of Tb(III) on signal intensity. Ce(IV), $4.0 \times 10^{-4}$ mol/L; $Na_2SO_3$, $1.0 \times 10^{-3}$ mol/L; HCl, $1.1 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; voltage, −800 V; plasma concentration of ofloxacin, 450 ng/mL.

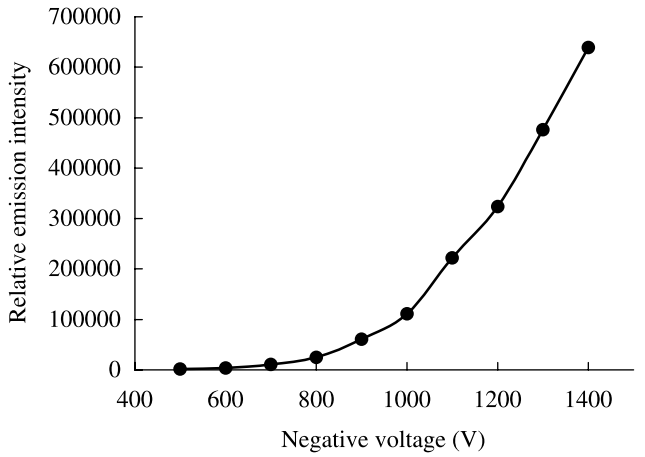


**Figure 12.** The relationship between CL intensity and the negative voltage. Ce(IV), $4.0 \times 10^{-4}$ mol/L; $Na_2SO_3$, $1.0 \times 10^{-3}$ mol/L; HCl, $1.1 \times 10^{-4}$ mol/L; Tb(III), $8.0 \times 10^{-4}$ mol/L; flow rate, 1.8 mL/min; plasma concentration of ofloxacin, 506 ng/mL.

limit was 7.5 ng/mL ($3\sigma$). It matched the requirements of *in vivo* analysis, as the plasma concentration of ofloxacin is in the range 100–5000 ng/mL after oral administration or injection (21).

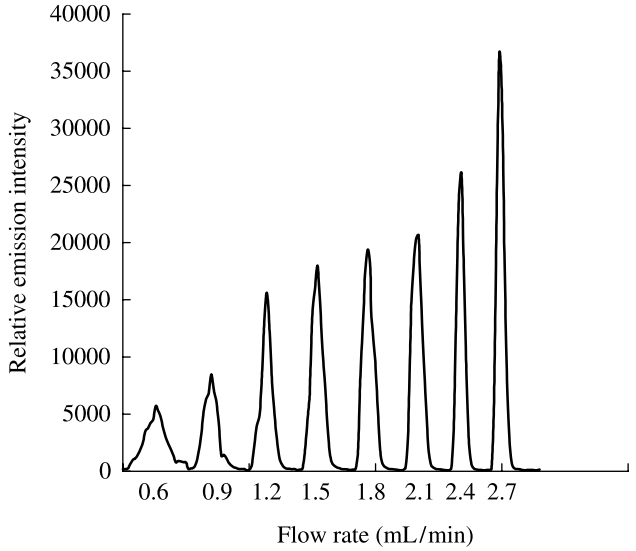


**Figure 13.** Influence of flow rate on CL intensity. Ce(IV), $4.0 \times 10^{-4}$ mol/L; $Na_2SO_3$, $1.0 \times 10^{-3}$ mol/L; HCl, $1.1 \times 10^{-4}$ mol/L; Tb(III), $8.0 \times 10^{-4}$ mol/L; voltage, –1100 V; plasma concentration of ofloxacin, 506 ng/mL.

## Precision and stability

Measurements were repeated at three concentration levels to test the precision (see Table 2). At all levels, the intra-day and inter-day RSD% were < 3.0% and < 7.0%, respectively. The stability of the method was also investigated. The plasma was frozen and thawed three times, then it was assayed. The results were compared with those obtained at room temperature—the RSD% for every level was < 3.0% (Table 2).

## Recovery

The added recovery was examined at 22.5, 225 and 675 ng/mL and the results are summarized in Table 3. The added recoveries were in the range 96.2–105.2% and this is satisfactory for pharmacokinetics studies. Extraction recovery was also calculated by comparing the emission intensity of the plasma sample with that of standard ofloxacin sample at the same concentration. The extraction recoveries at three concentration levels were tested. The results in Table 3 showed that although extraction recovery was moderate at low concentration, extraction recoveries were up to 86.4% and 81.9% at medium and high concentrations, respectively.

**Table 1. Analytical parameters for calibration**

| Sample | Linear range (ng/mL) | Linear regression equation ($I = aC + b$) | $r$ |
|---|---|---|---|
| Plasma sample of ofloxacin | 22.5–225 | $I = 1177.45C - 1296.2$ | 0.9994 |
| | 225–900 | $I = 514.7C + 167570$ | 0.9963 |

$I$, intensity; $C$, concentration.

**Table 2. Precision and stability of ofloxacin in human plasma**

| Samples | Precision (RSD%) Intra-day ($n = 11$) | Precision (RSD%) Inter-day ($n = 3$) | Stability (RSD%) |
|---|---|---|---|
| Low level (22.5 ng/mL) | 2.41 | 6.59 | 1.57 |
| Medium level (225 ng/mL) | 0.84 | 5.58 | 0.82 |
| High level (675 ng/mL) | 0.55 | 1.55 | 2.82 |

**Table 3. Recoveries of ofloxacin in human plasma**

| Sample | Added (ng/mL) | Found (ng/mL) | Added recovery (%) | Extraction recovery ($H_{Standard}$:$H_{Sample}$)* (%) |
|---|---|---|---|---|
| Plasma sample of ofloxacin | 22.5 | 21.6 | 96.2 | 53.1 |
| | 225 | 236.8 | 105.2 | 86.4 |
| | 675 | 669.7 | 99.2 | 81.9 |

*$H_{Standard}$, peak height of standard ofloxacin; $H_{Sample}$, peak height of plasma sample.

### Interference studies

The interference of most ions in plasma was studied. In our experiments, 10% of emission intensity variation was tolerable compared with ion-free samples. Plasma samples with 225 ng/mL ofloxacin containing various foreign ions were assayed. The results indicated that 1000-fold $K^+$, $NH_4^+$, $Na^+$, $Al^{3+}$, $H_2PO_4^-$, $NO_3^-$, $Cl^-$, $Ac^-$, $HCO_3^-$, 100-fold $Mg^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Zn^{2+}$, $Ba^{2+}$, $Ca^{2+}$, $SO_4^{2-}$ and dextrin had no interference. Hence, most of the ions had no interference, and the only pretreatment needed for plasma samples was deproteinization, which greatly simplified the assay procedure.

## CONCLUSIONS

By employing Ce(IV) as the oxidant, $Na_2SO_3$ as reductant and Tb(III) as sensitizer, the proposed method has been successfully applied to the determination of ofloxacin in human plasma. Experimental conditions have been optimized and the analytical characteristics determined. The method is a convenient tool, rapid (20 s/analysis) and well suited for pharmacokinetics studies, in which thousands of samples must be analysed daily. It was applied to pharmacokinetics studies of ofloxacin.

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