J Pharm Anal Vol 1, No 1, 46－50 (2011)

ORIGINAL ARTICLES

Rapid europium-sensitized fluorescent determination of ulifloxacin, the active metabolite of prulifloxacin, in human serum and urine

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Abstract: A new fluorescent method was developed based on the ulifloxacin-europium (Ⅲ)-sodium dodecylbenzene sulfonate system for the determination of ulifloxacin, the active metabolite of prulifloxacin. Sodium dodecylbenzene sulfonate formed a ternary complex with ulifloxacin-europium (Ⅲ) and significantly enhanced the characteristic fluorescence of europium (Ⅲ). The enhanced fluorescence intensity showed a good linear relationship with the concentration of ulifloxacin in the range of $5.0\times10^{-8}-2.0\times10^{-6}$ M with a detection limit of 2.0×10^{-10} M (3σ). This method is rapid and sensitive, and has been successfully applied to the determination of ulifloxacin in human urine and serum samples.

Keywords: ulifloxacin; prulifloxacin; europium; fluorescence; sodium dodecylbenzene sulfonate

1 Introduction

Prulifloxacin (PUFX, NM441, Figure 1), the prodrug of ulifloxacin (UFX, NM394, Figure 1), is a broad-spectrum oral fluoroquinolone antibacterial agent [1]. After oral administration and intestinal absorption, PUFX is rapidly metabolized by paraoxonases into UFX, the active metabolite of prulifloxacin [2-7]. Therefore, the *in vitro* antimicrobial activity was studied using ulifloxacin [6, 8,9].

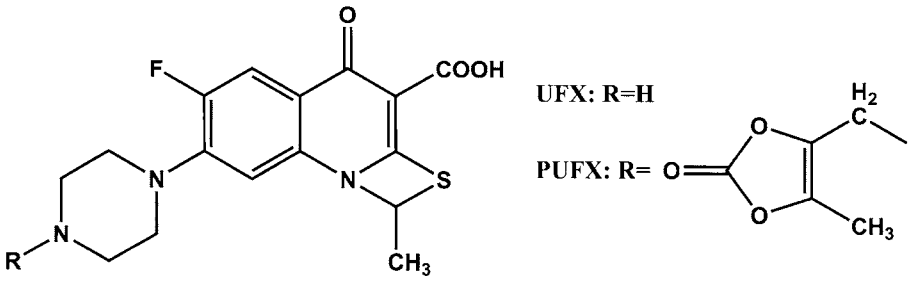

Figure 1 Chemical structures of ulifloxacin (UFX) and prulifloxacin (PUFX)

For the pharmacokinetic study of a prulifloxacin formulation product in healthy volunteers, a rapid, selective and sensitive analytical method is required to accurately determine the ulifloxacin concentration in body fluid . In previous studies, analysis of ulifloxacin has been performed by high performance liquid chromatography (HPLC) with ultraviolet(UV) detection [10], HPLC with fluorescence detection [11], HPLC-MS/MS [12], capillary zone electrophoresis [13], flow-injection chemiluminescence [14] and terbium-sensitized second-order scattering spectrofluorimetry [15].

Europium-sensitized fluorescent determination is an indirect method involving complexation of ligand molecules and europium ions. The intramolecular energy transfer from the absorbing ligand to the europium ions results in the emission of a strong narrow-band fluorescence with a large Stokes shift and long decay time. As a result, it could avoid the potential background fluorescent interferences from the biological matrix [16]. Quinolones and fluoroquinolones have suitable functional groups (carboxylic and keto-oxygen atoms) to form stable complexes with lanthanide ions. This technique has been widely utilized for the determination of quinolones norfloxacin [17], enoxacin [18], garenoxacin [19], ciprofloxacin [20], and trovafloxacin [21]. But the sensitivity is not very high as all the detection limits of these methods are over the range of 1.2×10^{-9} to 4.7×10^{-8} M. However, the europium-sensitized fluorescent determination method described here could provide a much higher sensitivity. The detection limit (3σ) could attain 2.0×10^{-10} M. Furthermore, there are few reports about the fluorescence mechanism. Compared with most of other approaches for the determination of UFX, the proposed method offers advantages in higher sensitivity, wider linear range, and better stability, which has been successfully applied to the determination of ulifloxacin in human urine and serum samples with satisfactory results, and the fluorescence enhancement mechanism is also discussed. To our knowledge, this is the first time that the europium-sensitized fluorescent determination is utilized for the determination of UFX.

Received 14 July 2010; Accepted 30 September 2010
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2 Materials and methods

2.1 Apparatus

Fluorescence intensity was measured on an Hitachi F-2500 fluorescence spectrophotometer (Japan), using a standard 10 mm path-length quartz cell, with 10 nm bandwidths for both the excitation and emission monochromators. Absorption spectra were measured on an UV-2401PC spectrophotometer (Shimadzu, Japan) equipped with 10 mm path-length quartz cells. The surface tension was measured on a Processor Tensiometer-K12 (KRÜSS Corp., Germany) with the precise degree of 0.01 mN/m by the Wilhelmy plate. The pH was measured using a Lei Ci pHs-3C pH-meter (Shanghai, China). Serum samples were centrifuged with a Xiang Yi TDZ4A-WS brachytely desk centrifuge (Changsha, China).

2.2 Reagents

A standard stock solution (1.0×10^{-3} M) of UFX (purity, 100.9%, Department of Pharmacy, Beijing Hospital, China) was prepared by dissolving 0.0349 g of UFX in dilute hydrochloric acid and then diluting to 100 mL with water. The solution was kept at 4 ℃ in the refrigerator and protected from light. Standard working solutions were prepared by diluting the standard stock solution with water.

A solution of europium (Ⅲ) (Eu^{3+}) (1.0×10^{-3} M) was prepared by dissolving 0.0176 g Eu_2O_3 (purity, 99.99%) in hydrochloric acid solution (1 : 1) and evaporating the solution to near dryness before diluting to 100 mL with water. The solution was kept at 4 ℃. The working solutions were diluted with water.

A sodium dodecylbenzene sulfonate (SDBS) solution (2.0×10^{-3} M) was prepared by dissolving 0.0774 g SDBS (purity, 90.0%) in water and then diluting to 100 mL with water.

A NH_4Cl-$NH_3\cdot H_2O$ buffer solution (pH 8.6, 0.1 M) was prepared by dissolving 1.3508 g NH_4Cl in water to 250 mL and then adjusting pH to 8.6 with $NH_3\cdot H_2O$.

All reagents used were of analytical grade unless otherwise indicated. Redistilled water made in-house was used throughout the study.

2.3 Methods

Various solutions were added (to a 10 mL volumetric flask) according to the following order: 0.5 mL of 1.0×10^{-4} M Eu^{3+} solution, an aliquot of UFX standard working solution, 0.5 mL of 2.0×10^{-3} M SDBS solution, and 1.0 mL of 0.1 M NH_4Cl-$NH_3\cdot H_2O$ buffer solution (pH 8.6). The resultant mixture was diluted to the volume with water and mixed thoroughly by shaking, and then allowed to stand for 15 min at room temperature. Final UFX concentrations were in the range of $5.0\times10^{-8}-2.0\times10^{-6}$ M. The fluorescence intensity was measured in a 10 mm path-length quartz cell with excitation and emission wavelengths of 276 nm and 616 nm, respectively.

2.4 Preparation of serum and urine samples

1.0 mL of each serum sample was deproteinized with 2.0 mL of acetonitrile, followed by vortexing for 5 min and centrifuging for 5 min at 1 500 × *g* [19]. No sample pretreatment was required for urine samples. Further dilution with water was made to ensure that the concentrations of the drug in the sample solutions fall within the linear range of the method.

3 Results and discussion

3.1 Spectral characteristics

The excitation and emission spectra of UFX (1, 1'), Eu^{3+} (2, 2'), UFX-Eu^{3+} (3, 3'), and UFX-Eu^{3+}-SDBS (4, 4') system are shown in Figure 2. The excitation spectrum of the UFX-Eu^{3+}-SDBS system exhibited a peak at 276 nm, which was selected as the excitation wavelength. From the emission spectra, no characteristic fluorescence of Eu^{3+} was observed in the Eu^{3+} system (2'), while weak characteristic fluorescence of Eu^{3+} was observed in the UFX-Eu^{3+} system (3'). Curve 4' is the emission spectrum of UFX-Eu^{3+}-SDBS system and strong emission peaks were observed at 594 and 616 nm, corresponding to transitions of the Eu^{3+} $^5D_0$-$^7F_1$ and $^5D_0$-$^7F_2$, respectively. Compared with the UFX-Eu^{3+} system, the fluorescence intensity(I_F) was enhanced about 37-fold by the addition of SDBS. Thereby, 276 nm and 616 nm were chosen as the excitation wavelength and emission wavelength, respectively.

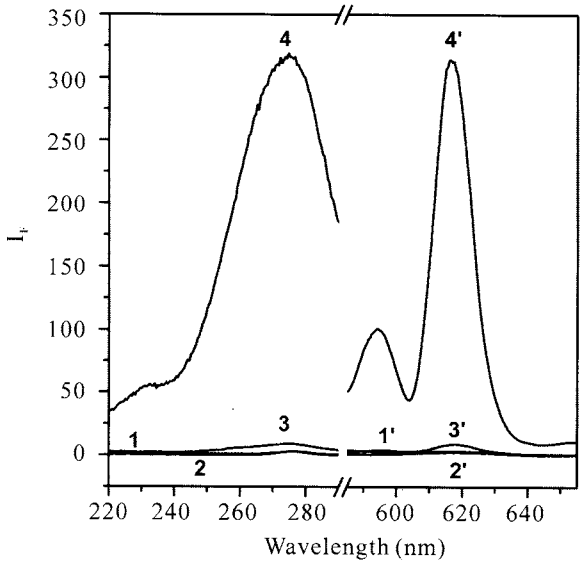

Figure 2 Excitation spectra and emission spectra. Experimental conditions: UFX, 5.0×10^{-6} M; Eu^{3+}, 5.0×10^{-6} M; SDBS, 1.0×10^{-4} M; NH_4Cl-$NH_3\cdot H_2O$, 0.01 M, pH = 8.6. 1, 1', UFX; 2, 2', Eu^{3+}; 3, 3', UFX-Eu^{3+}; 4, 4', UFX-Eu^{3+}-SDBS.

In order to study the interaction between UFX-Eu^{3+} and SDBS, the ultraviolet absorption spectra were recorded (Figure 3), which showed that the absorption peak of UFX-Eu^{3+} was at 267 nm. However, the absorbance of UFX-Eu^{3+} increased apparently after the addition of the SDBS solution, which was in accordance with the fluorescence enhancement of the excitation spectra in the UFX-Eu^{3+}-SDBS system (Figure 2). Also, the maximum absorption wavelength underwent a red shift from 267 nm to 273 nm, indicating the formation of a UFX-Eu^{3+}-SDBS ternary complex [22].

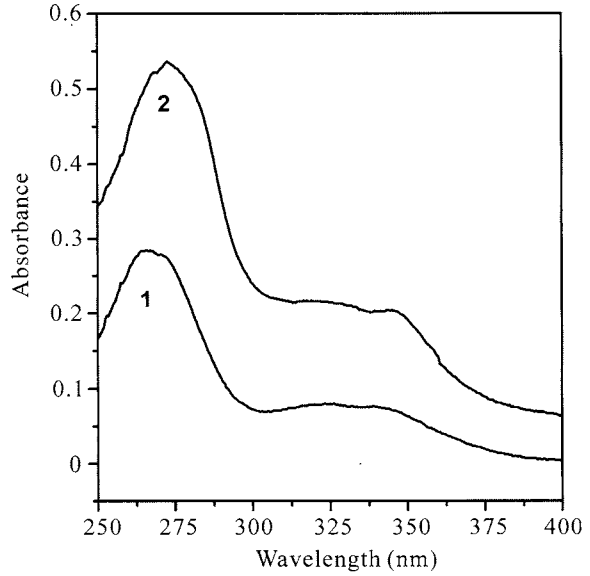

Figure 3 Ultraviolet absorption spectra. Experimental conditions: UFX, 1.0×10^{-5} M; Eu^{3+}, 4.0×10^{-5} M; SDBS, 1.0×10^{-4} M; NH_4Cl-$NH_3\cdot H_2O$, 0.01 M, pH=8.6. 1, UFX-Eu^{3+}; 2, UFX-Eu^{3+}-SDBS.

3.2 Effects of pH and buffers

The effect of pH on the fluorescence intensity of the system was studied in the range of 5.8 – 10.5. The results indicated that the optimal pH value was in the range of 8.2 – 8.8 (Figure 4), so pH 8.6 was selected for the recommended procedure. Setting pH at 8.6, the effect of the following buffer solutions on the fluorescence intensity was then examined, including NH_4Ac-$NH_3\cdot H_2O$, NaAc-HAc, NH_4Cl-$NH_3\cdot H_2O$, Tris-HCl, and KH_2PO_4-NaOH. It was found that UFX-Eu^{3+}-SDBS fluorescence system in the NH_4Cl-$NH_3\cdot H_2O$ medium of the concentration 0.01 M gave the highest sensitivity. Therefore, a 0.01 M NH_4Cl-$NH_3\cdot H_2O$ buffer solution of pH 8.6 was chosen for further study.

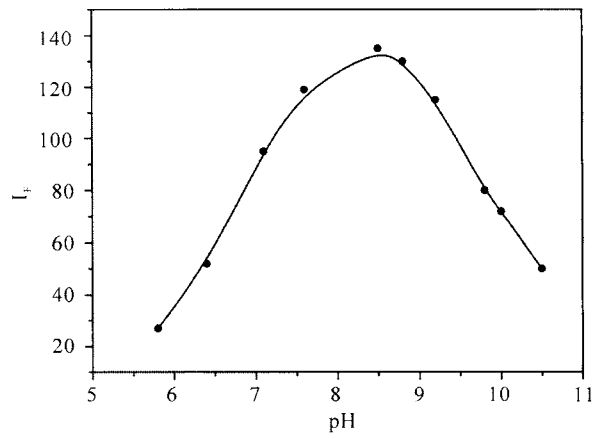

Figure 4 Effect of pH. Experimental conditions: UFX, 5.0×10^{-6} M; Eu^{3+}, 5.0×10^{-6} M; SDBS, 1.0×10^{-4} M.

3.3 Effect of europium (Ⅲ) concentration

With a fixed UFX concentration of 5.0×10^{-6} M, the effect of Eu^{3+} concentration on the fluorescence intensity of the system was investigated (Figure 5). Fluorescence intensity reached a maximum value with a Eu^{3+} concentration of 5.0×10^{-6} M. Therefore, the Eu^{3+} concentration of 5.0×10^{-6} M was selected for further experiments. ΔI_F was the net fluorescence intensity, with the background signal being subtracted.

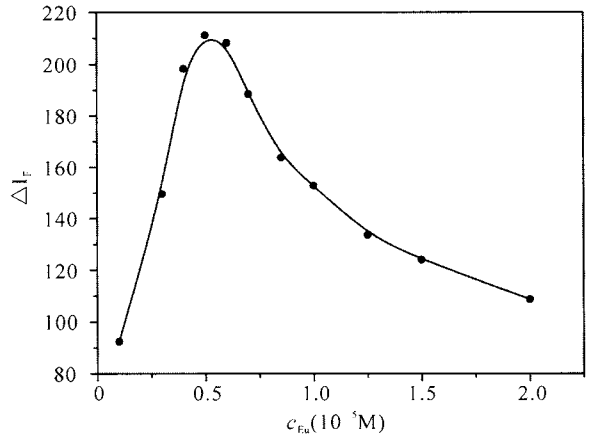

Figure 5 Effect of Eu^{3+} concentration. Experimental conditions: UFX, 5.0×10^{-6} M; SDBS, 1.0×10^{-4} M; NH_4Cl-$NH_3\cdot H_2O$, 0.01 M, pH=8.6.

3.4 Effect of surfactants

In order to investigate the effect of micellar media on the fluorescence intensity, various types of surfactants including CTAB (Cetrimonium bromide, cationic), GA (Arabic gum, non-ionic), OP (Polyoxyethylene nonylphenol ether, non-ionic), β-CD (β-cyclodextrin, non-ionic), Triton X-100 (non-ionic), Pluronic F68 (non-ionic), PEG40 (non-ionic), SDS (Sodium dodecyl sulfate, anionic), and SDBS (anionic) were studied (Table 1). It can be seen that there was no significant fluorescence enhancement in the presence of cationic surfactants, non-ionic surfactants or anionic surfactant SDS. However, a significant increase in fluorescence was observed in the presence of anionic surfactant SDBS. The effect of the SDBS concentration on the fluorescence intensity was also investigated (Figure 6). Maximum fluorescence intensity was observed at an SDBS concentration of 1.0×10^{-4} M, which was chosen for further experiments.

The mechanism of SDBS effect was studied by investigating the changes of surface tension of the fluorescence system with an increase of the SDBS concentration (Figure 7). It can be seen that the surface tension first decreased sharply, and then reached equilibrium at the SDBS concentration of 7.0×10^{-5} M, which can be considered as the apparent critical micelle concentration (CMC) of SDBS in this system. Since the SDBS concentration selected for the study (1.0×10^{-4} M) was well above the CMC, it could be concluded that the formation of micelles had a great impact on the enhancement of the fluorescence intensity of the system.

Table 1 Effects of different surfactants

Surfactants	Optimal concentration	Relative fluorescence intensity (%)
Cetrimonium bromide (CTAB)	3.0×10^{-4}M	6.2
β-cyclodextrin (β-CD)	2.0×10^{-3}M	0
Polyoxyethylene nonylphenol ether (OP)	10% (v/v)	0
Triton X-100	10% (v/v)	0
Arabic gum (GA)	3.0 g/L	3.8
Pluronic F68	2.5 g/L	2.7
PEG40	3.0 g/L	5.4
Sodium dodecyl sulfate (SDS)	1.0×10^{-4}M	4.7
Sodium dodecylbenzene sulfonate (SDBS)	1.0×10^{-4}M	100.0

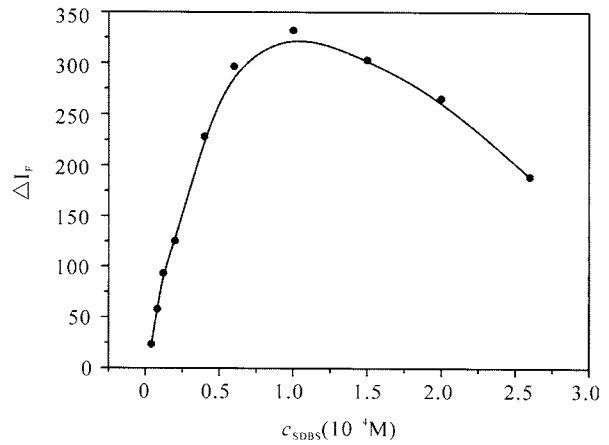

Figure 6 Effect of SDBS concentration. Experimental conditions: UFX, 5.0×10^{-6}M; Eu^{3+}, 5.0×10^{-6}M; NH_4Cl-$NH_3\cdot H_2O$, 0.01 M, pH=8.6.

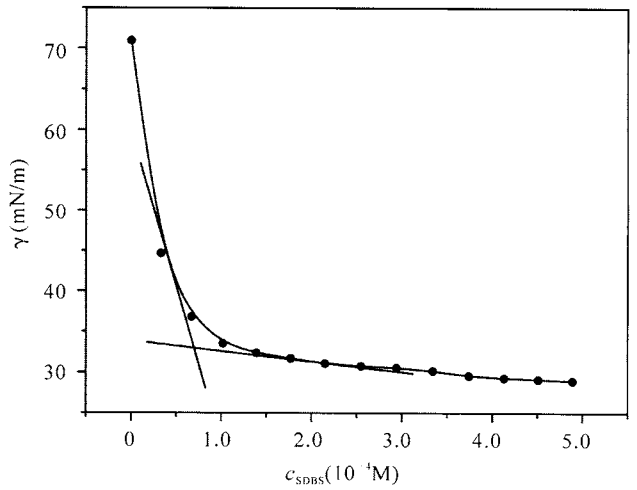

Figure 7 Effect on the solution surface tension with the addition of SDBS. Experimental conditions: UFX, 5.0×10^{-6}M; Eu^{3+}, 5.0×10^{-6}M; NH_4Cl-$NH_3\cdot H_2O$, 0.01 M, pH=8.6.

3.5 Effect of reagent addition sequence and fluorescence stability

The effect of the reagent addition sequence on the fluorescence intensity was studied. The results showed that the addition sequence of Eu^{3+}, UFX, SDBS, and NH_4Cl-$NH_3\cdot H_2O$ provided the optimal performance. Therefore, this addition sequence was chosen for further studies. The experiments also demonstrated that the fluorescence intensity of the system reached a maximum within 15 min after all the reagents had been added and remained stable for at least 3 h.

3.6 Effect of interfering species

The interference of metal ions, glucose, and amino acids which are commonly presented *in vivo* was studied by adding different amounts of these compounds to a 5.0×10^{-6}M UFX solution. The maximum permissible concentrations of the interfering substances causing a ±5% relative error in the fluorescence intensity are shown in Table 2. The results indicated that most species except for Cu^{2+} and Fe^{3+} had little effect on the fluorescence intensity of the system.

Table 2 Maximum permissible concentration of interference species

Interference species	Maximum permissible concentration (M)	Change of ΔI (%)
Fe^{3+}, Cl^-	1.0×10^{-7}	5.0
Cu^{2+}, SO_4^{2-}	4.0×10^{-7}	4.1
Vitamin C	1.5×10^{-6}	−4.8
Zn^{2+}, SO_4^{2-}	5.0×10^{-6}	−4.6
L-Methionine	2.0×10^{-5}	−4.7
Al^{3+}, Cl^-	5.0×10^{-5}	5.0
L-Histidine	1.0×10^{-4}	−4.6
NH_4^+, Cl	1.5×10^{-4}	−4.4
Mg^{2+}, Cl^-	2.0×10^{-4}	4.5
Ca^{2+}, Cl^-	3.0×10^{-4}	4.2
L-Glutamine	4.0×10^{-4}	−4.8
L-Cysteine	1.0×10^{-3}	4.3
Glycine	1.0×10^{-3}	−4.2
Glucose	1.5×10^{-3}	−4.5
Na^+, Cl^-	1.0×10^{-2}	4.4
K^+, Cl^-	1.0×10^{-2}	4.6

3.7 Analytical application

3.7.1 Linear range and detection limit

Under the optimal experimental conditions, the enhanced fluorescence intensity of the system (ΔI_F) responded linearly to the UFX concentration in the range of 5.0×10^{-8} – 2.0×10^{-6}M with a correlation coefficient of 0.9990. The detection limit for UFX calculated from the standard deviation of the blank (the reagent blank without UFX, $n=20$) (3σ) is 2.0×10^{-10}M.

In comparison with other methods reported, the rapid and simple method proposed in this paper offers significantly increased sensitivity (Table 3).

3.7.2 Recovery assay

The maximum concentration of UFX in the serum is between 1.0 μg/mL (2.8×10^{-6}M) and 1.6 μg/mL (4.6×10^{-6}M) after a single oral administration of 300, 450 and 600 mg PUFX. In contrast, the concentration of UFX in urine is very high after dosing, and can reach more than 60 μg/mL (1.7×10^{-4}M) [8].

In order to ensure that the concentrations of the drug fall within the linear range of the method, urine samples were

diluted 1 000-fold with water and then analyzed by standard addition method, and serum samples were deproteinized first and then diluted 40-fold prior to analysis. The results of spiked urine and serum samples are listed in Table 4.

Table 3 Comparison of methods for the determination of UFX

Method	Detection limit (M)	Linear range (M)	Application	References
HPLC	1.7×10^{-8}	$1.4\times10^{-8}-1.7\times10^{-6}$	Aqueous human humor	[10]
HPLC-Flu		$2.9\times10^{-8}-2.9\times10^{-6}$	Human plasma	[11]
HPLC-MS		$7.2\times10^{-8}-1.4\times10^{-5}$	Human plasma	[12]
Capillary zone electrophoresis		$5.7\times10^{-8}-5.7\times10^{-6}$	Human plasma	[13]
Chemiluminescence	5.5×10^{-9}	$1.0\times10^{-8}-5.0\times10^{-6}$	Spiked human serum and urine	[14]
Terbium-sensitized second-order scattering spectrofluorimetry	3.9×10^{-9}	$2.0\times10^{-8}-1.0\times10^{-5}$	Spiked human serum and urine	[15]
Europium probe spectrofluorimetry	2.0×10^{-10}	$5.0\times10^{-8}-2.0\times10^{-6}$	Spiked human serum and urine	Present method

Table 4 Recovery of UFX in spiked human urine and serum samples ($n=5$)

Sample	Added (M)	Found (M)	Recovery ± RSD (%)
Urine 1	2.5×10^{-7}	2.6×10^{-7}	104.0 ± 0.1
Urine 2	3.8×10^{-7}	3.8×10^{-7}	100.0 ± 2.7
Serum 1	2.0×10^{-7}	2.1×10^{-7}	105.0 ± 3.0
Serum 2	3.0×10^{-7}	3.0×10^{-7}	100.0 ± 2.3
Serum 3	4.0×10^{-7}	3.9×10^{-7}	97.5 ± 2.9

4 Conclusion

A new europium-sensitized fluorescence method for the determination of UFX is proposed here. Under optimal conditions, the enhanced fluorescence intensity is in proportion to the UFX concentration over the range of $5.0\times10^{-8}-2.0\times10^{-6}$ M with a detection limit (3σ) of 2.0×10^{-10} M. In comparison with most of methods reported for the determination of UFX, the proposed method is faster and simpler, and has higher sensitivity. It has been successfully applied for the determination of UFX in human urine and serum samples with minimal sample pretreatment. The research on fluorescence mechanism reveals that the formation of micelles plays an important role in the fluorescence enhancement in the complex system studied.

Acknowledgments

This project was supported by the National Natural Science Foundation of China (Nos. 20875056, 20775043). The authors would like to thank the Key Laboratory of Education Ministry on Colloid & Interface Chemistry, Shandong University, for kindly providing Processor Tensiometer-K12 for the determination of surface tension.

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