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ORIGINAL PAPER

Determination of Ofloxacin using a Highly Selective Photo Probe Based on the Enhancement of the Luminescence Intensity of Eu^{3+}—Ofloxacin Complex in Pharmaceutical and Serum Samples

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Abstract A rapid, simple and sensitive spectrofluorimetric method for determination of trace amount of ofloxacin was developed. At pH 5.1 the ofloxacin enhances the luminescence intensity of the Eu^{3+} ion in Eu^{3+}- ofloxacin complex at λ_{ex}=365 nm. The produced luminescence intensity of Eu^{3+}-ofloxacin complex was in proportional to the concentration of ofloxacin. The working range for the determination of ofloxacin was 5.0×10^{-9}–5.0×10^{-6} mol L^{-1} with lower detection limit (LOD) and quantitative detection limit (QDL) of 3×10^{-9} and 9×10^{-9} mol L^{-1}, respectively. The enhancement mechanism of the luminescence intensity in the Eu^{3+}-ofloxacin system has been also explained. The method revealed good selectivity for ofloxacin in the presence of coexisting substances and used successfully for the assay of ofloxacin in pharmaceutical preparations and serum. A comparison with other standard methods was also discussed.

Keywords Ofloxacin · Europium · Luminescence · Enhancement · Energy transfer · Photo probe

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Introduction

Ofloxacin (OFLX) [9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1 piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxacine-6-carboxylic acid], (Fig. 1), is a quinolone synthetic antibiotic, which acts against resistant mutants of bacteria by inhibiting DNA gyrase [1]. Ofloxacin possesses two relevant ionizable functional groups: a basic piperazinyl group and a carboxylic acid group. The carboxylic and the carbonyl groups are required for antimicrobial activity, and also, being a susceptible convenient environment for coordinating to various metal ions and it is in these groups that the chelation interaction with various cations takes place. A potential problem with the increasing use of quinolone-type antibiotics for the treatment of systematic illness is their chelation with several cations and such interaction could be a reduction in the bioavailability and effectiveness of the quinolone compounds. Quinolones also effect trace metal metabolism and are potent inhibitors of some copper-and zinc-dependent enzymes [2]. Several methods for the determination of OFLX in pharmaceuticals or/and in human urine have been reported in the literature including spectrophotometric [2–6], chemiluminescence [7, 8], chromatographic [9–13], electrophoretic [14–16] and electroanalytic [17–21]. In this paper, a novel spectrofluorimetric method was developed for measuring the concentration of OFLX in pharmaceutical formulation and serum samples. The OFLX concentration was determined by the complexation between the OFLX as a ligand and the Eu^{3+} ion where the possibility of energy transfer and the sensitization of the Eu^{3+} ion characteristic luminescence bands by OFLX antenna is established and investigated. The results reveal that the luminescence intensity of Eu^{3+} ion is a function of OFLX concentration which in turn offers a hypersensitive technique for OFLX assessment.

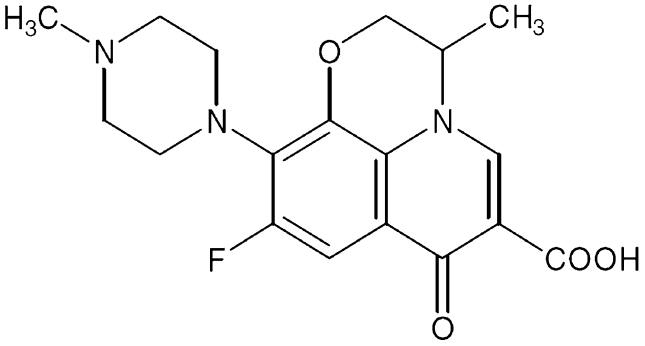


Fig. 1 Chemical structure of OFLX

The absorption and emission spectra of OFLX and Eu^{3+}–OFLX complex were measured in acetonitrile at pH 5.1. In comparison with other spectrofluorimetric techniques [22–24], this method is simple, relatively interference free from coexisting substances and can successfully be applied to the determination of OFLX in pharmaceutical formulations and in serum samples with remarkably satisfactory results.

Experimental

Materials

Pure standard OFLX supplied by the National organization for Drug control and Research (Giza, Egypt). Pharmaceutical preparation of Occufloxin eye drops containing 0.3% of OFLX produced by The Nile Co. for Pharmaceuticals and chemical Industries; Egypt is purchased from local market.

Reagents

All chemicals used are of analytical grade and pure solvents were purchased from (Aldrich). A stock solution of OFLX (10^{-2} mol L^{-1}) was freshly prepared by dissolving 90.300 mg in 25 ml pure methanol. More diluted solution (3×10^{-4} mol L^{-1}) was prepared by appropriate dilution with acetonitrile. Stock and working solutions are stored at 0–4 °C when are not in use.

Stock solution (10^{-2} mol L^{-1}) of Eu^{3+} ion was prepared by dissolving 87.900 mg $Eu(NO_3)_3$ (delivered from Aldrich- 99.99%) in small amount of ethanol in 25 ml measuring flask, then dilute to the mark with ethanol. The working solution of Eu^{3+} ion of 1×10^{-4} mol L^{-1} is obtained by appropriate dilution with acetonitrile. The pH 5.1 was adjusted by using acetate buffer.

Apparatus

All luminescence measurements are carried out on Shimadzu RF5301 (PC) spectrofluorophotometer in the range 290–750 nm. The absorption spectra are recorded with a Unicam UV-Visible double beam spectrophotometer from Helios. The spectrophotometer is equipped with a temperature-controller cell holder. All pH measurements are made with a pHs-JAN-WAY 3040 ion analyzer.

General Procedure

Solutions were prepared in 10 mL measuring flasks, where 3×10^{-4} mol $L^{-1}$of OFLX solution and 1×10^{-4} mol $L^{-1}$of Eu^{3+} solution were mixed then the mixture was diluted to the mark with acetonitrile and was adjusted at pH 5.1 by using 0.1 mol L^{-1} of acetic acid -sodium acetate solutions; and was stored for 10–15 min at room temperature. The above procedure was used for the subsequent measurements of absorption, emission spectra and effect of time, pH and solvents. The luminescence intensity is measured at $\lambda_{ex}/\lambda_{em}$ =365/617 nm.

Determination of OFLX in Pharmaceutical Preparations

Appropriate volume was taken from Occufloxin eye drops containing 0.3% of OFLX (8×10^{-2}mol L^{-1}) to obtain 3×10^{-4} mol L^{-1} in 25 mL acetonitrile then further dilution to the linear range concentration. Solutions were standing for about 10–15 min. The concentration of the drug was determined by using 9 concentrations for each sample from the corresponding calibration graph.

Determination of OFLX in Serum Solution

Three milliliter of citrate solution was added to 4.0 mL plasma of a real health volunteer and 1.0 mL human serum was centrifuged for 15 min at 4000 rounds/minute to remove proteins, then 100 μl (micro liter) of the serum was added to 6 mL of acetonitrile in 10 mL measuring flask then 0.1 mL of Eu^{3+} ion was added to the solution and completed to the mark with acetonitrile. The luminescence intensity of the test solution was measured before and after addition of Eu^{3+} optical sensor. The change in the luminescence intensity was used for determination of OFLX in serum sample.

Results & Discussions

Spectral Characteristics

The absorption spectrum of 3×10^{-4} mol L^{-1} of OFLX in acetonitrile shows two bands in UV at 298 and 335 nm with molar absorptivity coefficient ($\varepsilon=2.87\times10^{5}$ and 2.33×10^{5} $M^{-1}L$ cm^{-1}), respectively. Upon addition of the lanthanide ion (Eu^{3+}), a blue shift was observed in the two bands by 2 nm, as shown in Fig. 2. The ion titration revealed that the complex formed with M : L ratio (1: 3) Fig. 3, which

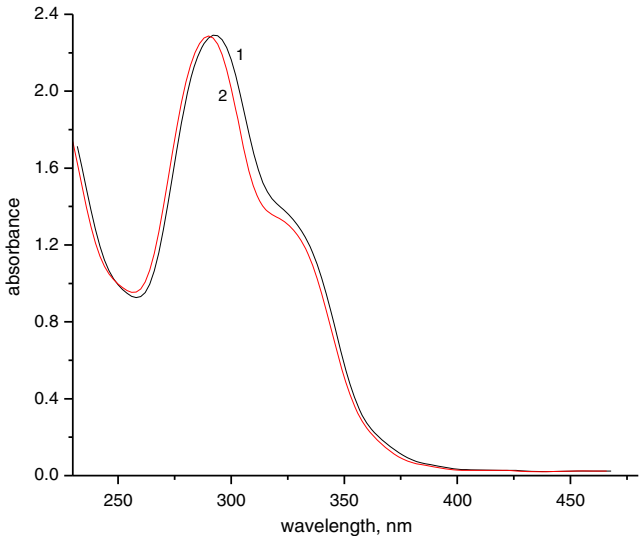


Fig. 2 Absorption spectra of (1)- 3×10^{-4} mol L^{-1} of OFLX (2)- 3×10^{-4} mol L^{-1} of OFLX with 1×10^{-4} mol L^{-1} of Eu^{3+} in acetonitrile

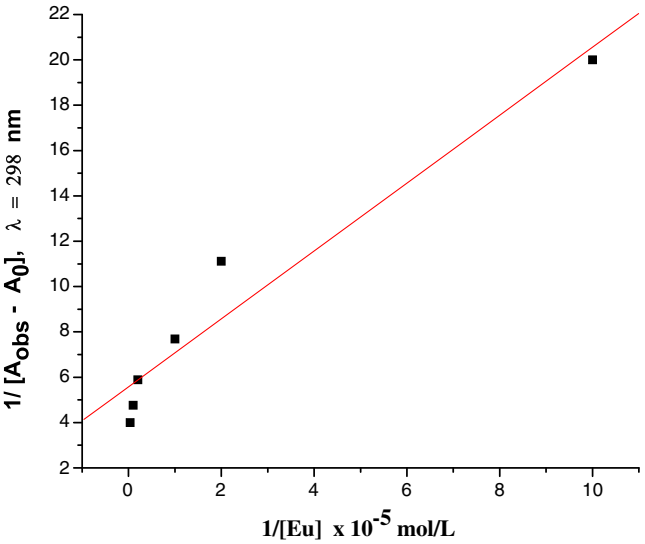


Fig. 4 Estimation of the apparent formation constant (K_{for}) of Eu^{3+} with OFLX complex in Acetonitrile using Benesi-Hildebrand -type plot

indicates that the ligand may coordinate to the metal via different coordination sites. The estimation of the apparent formation constant (K_{for})=3.72×10^5 mol/L of Eu^{3+} with OFLX complex in acetonitrile using Benesi-Hildebrand -type plot Eq. [25], Fig. 4.

$$\frac{1}{A_{obs}-A_o}=\frac{1}{A_c-A_o}+\frac{1}{K_{for}\,(A_c-A_o)[M]}$$

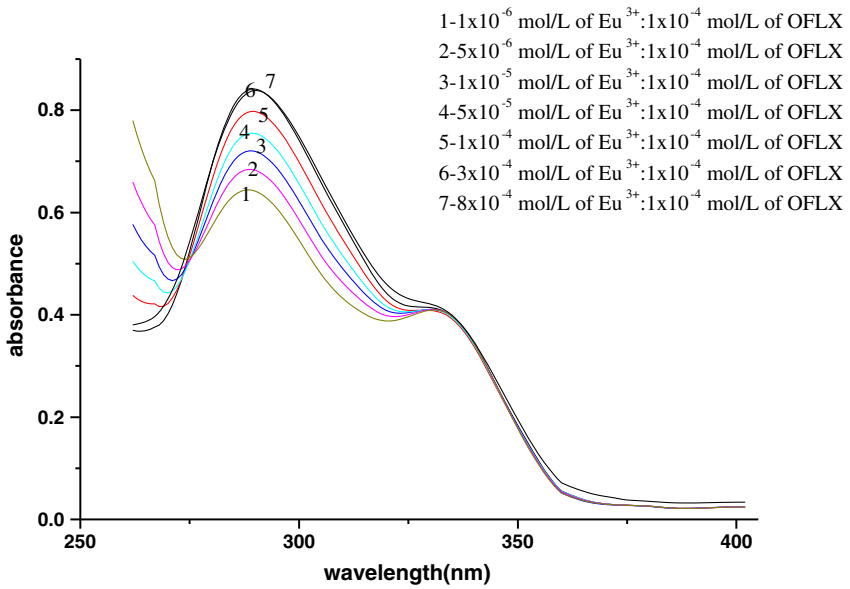
Where A_o, A_c, A_{obs}, K_{for} and [M] are the absorbance of the ligand, the absorbance of the complex, the absorbance of the ligand at various concentrations of the metal ion, the formation constant and the concentration of the metal ion, respectively.

The excitation and emission spectra of Eu^{3+} ion in Eu^{3+}-OFLX complex dissolved in acetonitrile at λ_{ex}=365 nm and λ_{em}=617 nm, respectively, were shown in Fig. 5. The emission bands are attributed to different transitions from ($^5D_0\rightarrow{}^7F_0$= 580, $^5D_0\rightarrow{}^7F_1$=590, $^5D_0\rightarrow{}^7F_2$=617, $^5D_0\rightarrow{}^7F_3$=650, $^5D_0\rightarrow{}^7F_4$=690 and $^5D_0\rightarrow{}^7F_5$=710 nm), respectively.

Effect of pH

The pH of the medium has great effect on the luminescence intensity of the system. The experimental results showed that the luminescence intensity reached maximum at pH 5.1, Fig. 6. Therefore acetate buffer solutions of pH 5.1

Fig. 3 Absorption spectra of 3×10^{-4} mol L^{-1} of OFLX with different molar ratio of Eu^{3+} in acetonitrile



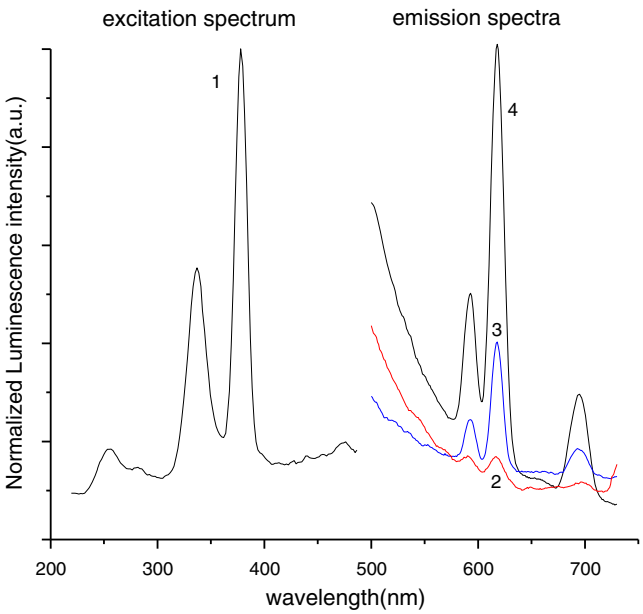


Fig. 5 Luminescence excitation spectrum (1) and emission spectra of 1×10^{-4} mol/L of Eu^{3+} with different concentrations of OFLX (2, 3, 4) in acetonitrile at λ_{ex}=365 nm

containing 0.1 mol L^{-1} acetic acid/sodium acetate were prepared and used throughout this study.

Effect of Time

The effect of time on the chelation reaction of the Eu^{3+}-OFLX system at room temperature was studied, it has been found that the reaction needs 10 min to reach maximum intensity. The luminescence intensity then remained constant for at least 2 h; therefore all measurements are made within 2 h during the study.

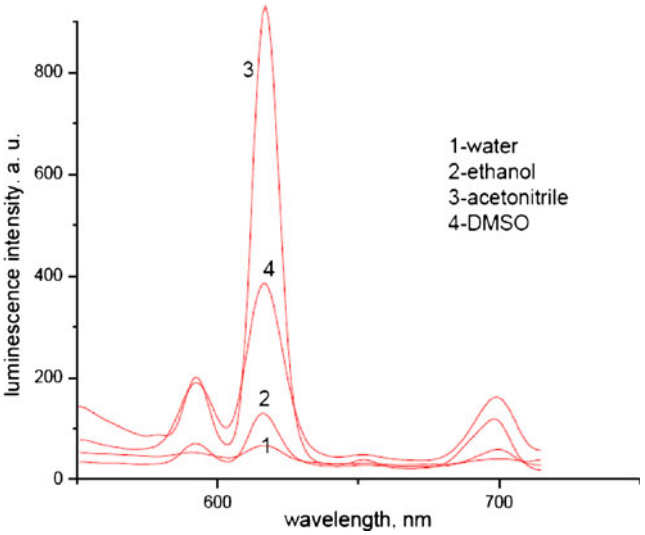


Fig. 7 Luminescence spectra of 1×10^{-4} mol L^{-1} of Eu^{3+} with 3×10^{-4} mol L^{-1} of OFLX in different solvents at λ_{ex}=365 nm

Effect of the Addition Order of Reagents

Addition of the reagents in different orders has great influence on the luminescence intensity. The experimental results indicate that the optimum luminescence intensity is obtained when solutions are added in the following order: OFLX, Eu^{3+} and buffer. So this order was chosen in the all experiments.

Effect of the Concentration of OFLX

The influence of OFLX concentration on the luminescence intensities of the Eu^{3+}-OFLX complex at constant concentration of Eu^{3+} (1×10^{-4} mol L^{-1}) was studied. It has been observed that luminescence intensity increases as the concentration of

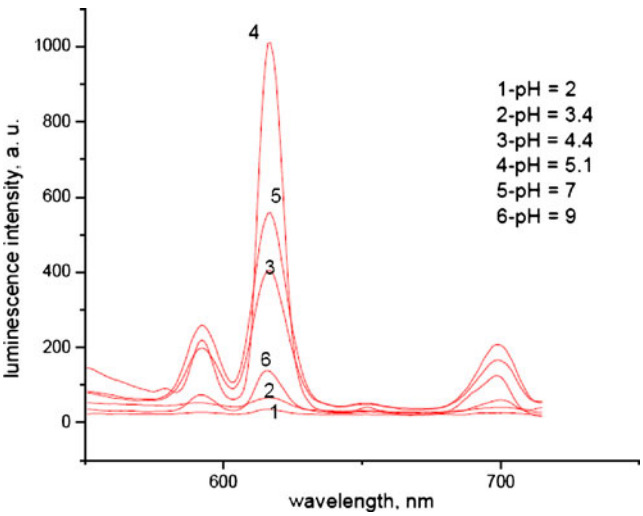


Fig. 6 Luminescence spectra of 1×10^{-4} mol L^{-1} of Eu^{3+} with 3×10^{-4} mol L^{-1} of OFLX in acetonitrile at λ_{ex}=365 nm in different pH

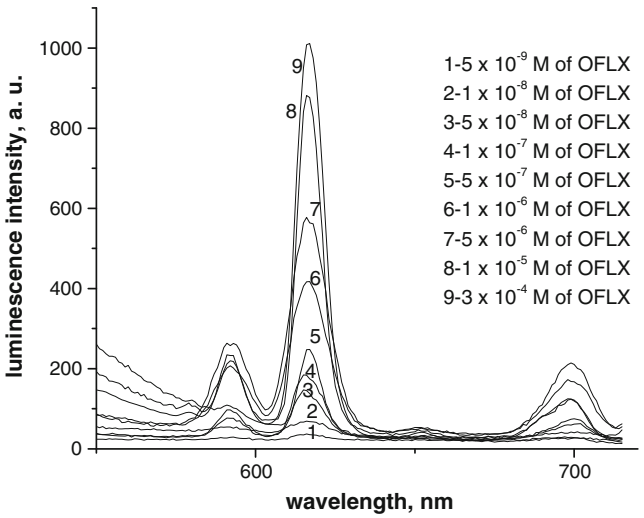


Fig. 8 Luminescence spectra of 1×10^{-4} mol L^{-1} of Eu^{3+} in acetonitrile at λ_{ex}=365 nm in different concentrations of OFLX

Table 1 Sensitivity and regression parameters for optical sensor

Parameter	Method
λ_{em}, nm	617
Linear range, mol L^{-1}	5×10^{-9}–5×10^{-6}
Limit of detection (LOD), mol L^{-1}	3.0×10^{-9}
Limit of quantification (LOQ), mol L^{-1}	9×10^{-9}
Regression equation, Y[a]	
Intercept (a)	64.5
Slope (b)	$9x10^{8}$
Standard deviation	1.54
Variance (Sa^2)	2.37
Regression coefficient (r)	9.99

[a] Y=a+bX, Where Y is luminescence intensity, X is concentration in n mol L^{-1}, a is intercept, b is slope

OFLX increases and reaches a maximum and remained constant when OFLX concentration is 3×10^{-4} mol L^{-1}.

Effect of the Concentration of Eu^{3+}

The influence of Eu^{3+} ion concentration on the luminescence intensities of the solutions containing 3.0×10^{-4} mol L^{-1} of OFLX was studied under the same experimental conditions shown above. The luminescence intensity increased with increasing the concentration of Eu^{3+} ion up to 1×10^{-4} mol L^{-1}, (i.e. At 1:3 composition ratio for the Eu^{3+} : OFLX system, the highest intensity of Eu^{3+} luminescence is achieved. At higher concentrations of Eu^{3+} ion, we noticed a marked decrease in the luminescence intensity of Eu^{3+} ion.

Effect of Solvent

The effect of the solvent on the luminescence intensities of the solutions containing 3.0×10^{-4} mol L^{-1} of OFLX and 1.0×10^{-4} mol L^{-1} Eu^{3+} was studied under the conditions optimized above. The high intensity of the luminescence of Eu^{3+}-OFLX solution was observed in aprotic solvents like acetonitrile and DMF as shown in Fig. 7. This may be attributed to the formation of anhydrous solvates of Eu^{3+}-OFLX complex introducing solvent molecules in the first coordination sphere of Eu^{3+}-OFLX complex leading to the enhancement of the intensity of the transitions ($^5D_0\rightarrow{}^7F_0$= 580, $^7F_1$=590, $^7F_2$=617, $^7F_3$=650, $^7F_4$=690 and $^7F_5$= 710 nm), especially $^5D_0\rightarrow{}^7F_2$=617 transition of the Eu^{3+} [26–28]. Also, the luminescence intensities for the complex in aprotic solvent like acetonitrile and DMF are stronger than in protic solvent like water and ethanol. This could be attributed to the efficient quenching of the excited state of the lanthanide ions by interactions with high-energy vibration oscillators like O-H groups [29].

Analytical Application

Linear Range and Limit of Detection

Under the experimental conditions, there is a linear relationship between luminescence intensity and OFLX concentration (Calibration Curve) in the range of 5.0×10^{-9} to 5.0×10^{-6} mol/L (1.806–1806 μg/L) with a correlation coefficient of 0.999.

The regression equation is luminescence intensity=$9\times10^{8}\times$Concentration (mol L^{-1})+64.5. Limits of detection (LOD) and quantitation (LOQ) are defined as 3**s/d** and 10**s/d**, respectively, [30] where **s** is its standard deviation and d is slope. LOD and quantitation (LOQ) are calculated at pH 5.1 to be 3×10^{-9} (1.084 μg/L) and 9×10^{-9} mol/L (3.24 μg/L), respectively. Fig. 8, see Table 1.

Robustness and Ruggedness

The robustness of the method was evaluated by making small incremental changes in the concentration of $Eu^{3+}$and OFLX and contact time, and the effect of the changes

Table 2 Method robustness and ruggedness expressed as intermediate precision (% RSD)

Method	Ofloxacin taken[a]	Robustness			Ruggedness
		Parameter altered			Inter-analysts, (%RSD) (n=3)
		Concentration of Eu^{3+}[b](%RSD)	Concentration of ofloxacin[b](%RSD)	Reaction time[b]	
Occufloxin eye drops containing 0.3% of ofloxacin produced by The Nile Co.	5	1.48	2.27	0.55	1.35
Serum sample	1.0	1.62	1.97	0.66	1.89

[a] The values are muiltiplied by 10^{-7} mol L^{-1}

[b] Concentrations of Eu^{3+} were 2, 5 and 6×10^{-4} mol L^{-1}; and the concentrations of ofloxacin were 4, 5 and 7×10^{-4} mol L^{-1}

[c] The reaction times studied were 10, 12 and 15 min

Table 3 Determination of (OFLX) in serum and pharmaceutical preparations using Eu^{3+} - (OFLX) optical sensor

Drug	Added (x 10^{-7} M)	Found (x 10^{-7} M)	Average[a]	Average recovery±R.S.D. (%)	B.P. (LC)
Occufloxin eye drops containing 0.3% of ofloxacin produced by The Nile Co.	1.5	1.52, 1.53, 1.47	1.49		
	2.3	2.32, 2.33, 2.27	2.29	96.3±0.44	97.5±1.0
	3.5	3.51, 3.52, 3.44	3.47		
Serum sample	2.0	1.92, 1.93, 1.92	1.92		
	4.0	4.21, 4.21, 4.20	4.21	101.2±0.42	96.3±0.4
	6.0	5.81, 5.81, 5.80	5.81		

[a] Average of nine measurements

was studied on luminescence intensity of the optical sensor. The changes had negligible influence on the results as revealed by small intermediate precision values expressed as% RSD (Relative Standard Deviation) (≤2.27%). Method ruggedness was expressed as the RSD of the same procedure applied by three different analysts. The inter-analysts RSD were within 1.89% for the same OFLX concentrations ranged from 1.35-1.89% suggesting that the developed method was rugged, the results are shown in Table 2.

Selectivity

The proposed method was tested for selectivity by placebo blank and synthetic mixture analysis. A placebo blank containing talc (200 mg), starch (200 mg), lactose (20 mg), calcium carbonate (50 mg), calcium dihydrogen orthophosphate (20 mg), methyl cellulose (40 mg), sodium alginate (50 mg), magnesium stearate (80 mg), norfloxacin (3×10^{-4} mol/L)and enrofloxacin (3×10^{-4} mol/L) were extracted with water and the solution made as described under "analysis of dosage forms". A convenient aliquot of solution was subjected to analysis according to the recommended procedures. In the method of analysis, there was no interference by the inactive ingredients.

A separate test was performed by applying the proposed method to the determination of OFLX in a synthetic mixture. To the placebo blank of similar composition, different amounts of OFLX of different products were added, homogenized and the solution of the synthetic mixture was prepared as done under "analysis of dosage forms". The filtrate was collected in a 100 mL flask. Five mL of the resulting solution was assayed (n=3) by proposed method which yielded a % recovery of 104.0–108.41±0.67 and 99.9±0.4 for eye drops and serum samples, respectively.

Recovery

The average recoveries of OFLX were evaluated at three concentration levels of (150, 230, and 350 n mol L^{-1}) each one was repeated three times and from peak intensity of

Table 4 Comparison of spectrofluorimetric methods for the determination of (OFLX)

Method	Linear range (μg/L)	Detection limit (μg/L)	References
Spectrophotometric	2500–25000	2500	[4]
Chemiluminescence	3–700	1.99	[8]
Chromatographic	50–2000	20	[9]
Electrophoretic	500–50000	200	[14]
	250–5000	250	[15]
	10000–100000	8500	[16]
Electroanalytic	79–197500	1.0	[17]
	7227–83115	1879	[18]
	7227–289096	1445	[19]
	3613–180685	108	[20]
			[21]
Spectrofluorimetric	25–500	8.0	[22]
	0.5–16.0	0.14	[23]
Spectrofluorometric method	1.806–1806	1.084	Present work

assayed samples comparison to the one of reference standards prepared in acetonitrile, then recoveries were calculated using the formula:

$$\%\,\text{Recovery} = \text{peak intensity serum}/\text{peak intensity acetonitrile} \times 100$$

The recommended procedure under "Calibration Curve" was performed. A blank experiment was carried out simultaneously. We determined the nominal content of OFLX using the following equation:

$$\text{Recovery}_{in\ vivo} = \text{Delivery}_{in\ vivo} \times \text{Recovery}_{in\ vitro}/\text{Delivery}_{in\ vitro}$$

This means that% recovery for OFLX in real human serum=Concentration of the drug in real serum X% recovery in spiked serum/Concentration of the drug in spiked serum .The results in Table 3 show that the method is successful for the determination of OFLX and that the excipients in the dosage forms did not interfere. The results obtained (Table 3) were statistically compared with the official British Pharmacopoeia [B.P] method [31]. The average recovery and R.S.D for the eye drops in our method were found to be (96.3% and 0.44%). and (101.2% and 0.42%) for serum sample. Data obtained by B. P method showing average recovery 97.3% and R.S.D 1.0% and 96.3% and R.S.D 0.4% for eye drops and serum samples, respectively, were also presented for comparison and show a good correlation with those obtained by the proposed method.

Determination of OFLX in Pharmaceutical Preparations and in Serum

The developed method is applied to the determination of OFLX in pharmaceutical preparations as shown in Table 3. For the assay of OFLX, the samples must be diluted appropriately within the linear range of determination of OFLX and the sample solution is analyzed by the method developed above, using the standard calibration method. The average recovery and relative standard deviation (RSD) are (96.3% and 0.44%) respectively. Data obtained by Liquid Chromatography method of British Pharmacopoeia [B.P. 2000] (average recovery 97.5% and R.S.D 1.0%) are also presented for comparison and show a good correlation with this obtained by the proposed method. The developed method can be easily performed and afforded good precision and accuracy when applied to the determination of OFLX in pharmaceutical preparations.

The developed method was also, applied to the determination of OFLX in human serum sample. Proteins in human serum interfere seriously for the system. Therefore; 1.0 ml serum was centrifuged for 15 min at 4000 r/min to remove proteins. Then 100 μl of the serum was added to 8.9 mL of acetonitrile, 1.0 mL buffer, added to a 0.1 mL of Eu^{3+} to serum, and analyzed by measuring the luminescence intensity as mentioned above. The experimental results in Table 3 show that an average recovery of 101.3% with relative standard of 0.42, which indicates that the developed method can be easily performed and affords good precision and accuracy when applied to human serum sample.

By comparison with some existing methods, as shown in Table 4, the present method has many advantages; it is high sensitive, good stability and wide linear range. It avoids potential background fluorescent emission interferences from the protein. So this method may provide a novel and viable kind of luminescent probes for the determination of biomolecular systems.

Conclusion

Based on the sensitization of the luminescence intensity of Eu^{3+} ion by the addition of OFLX and forming an optimized 1:3 Eu^{3+}:OFLX system in acetonitrile at pH 5.1, we proposed a simple, rapid and sensitive method for the determination of OFLX over a concentration range of 5×10^{-9} to 5.0×10^{-6} mol L^{-1} and detection limit of 3×10^{-9} mol L^{-1} . The developed method is selective and accurate for routine control analysis of the OFLX drug family. The proposed method is used usefully for the determination of OFLX in pharmaceutical formulations and in human serum samples.

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