

Determination of Heparin Using Norfloxacin-cerium Complex as a Fluorescence Probe by Spectrofluorimetry

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A simple, rapid, practical and sensitive spectrofluorimetric method was developed for the determination of trace amount of heparin (Hep). Under the optimum conditions, we studied the interaction between NFLX-Ce³⁺-Hep complex by using absorption and fluorescence spectra. It was observed that Hep remarkably enhance the fluorescence intensity of the NFLX-Ce³⁺ complex at $\lambda = 356$ nm in the buffer solution of pH = 7.60 and the enhancement effect is shown to relate with the concentration of Hep. The linear range and detection limit for the determination of Hep was obtained. By the Rosenthal graphic method, the association constant (K) and binding numbers (N) of Hep with probe were investigated. This method is relatively free of interference from coexisting substances and successfully applied for the determination of heparin in heparin sodium injection samples. A suitable mechanism of fluorescence enhancement between NFLX-Ce³⁺ and the NFLX-Ce³⁺-Hep systems were proposed and discussed.

Key Words: Heparin, Norfloxacin (NFLX), Cerium, Spectrofluorometric method

Introduction

Heparin (Hep) is a naturally-occurring anticoagulant produced by basophiles and mast cells, with a molecular weight ranging from 12000 - 15000 g/mol. It is a member of the glycosaminoglycan family of carbohydrates and consists of a variably-sulfated repeating disaccharide unit. The most common disaccharide unit is composed of a 2-O-sulfated iduronic acid and 6-O-sulfated, N-sulfated, glucosamine. A highly-sulfated glycosaminoglycan, Hep is widely used as an injectable anticoagulant. The whole Hep molecule is negatively charged in aqueous solution and the average charge¹ is -70. It can also be used to form an inner anticoagulant surface on various experimental and medical devices such as test tubes and renal dialysis machines. Pharmaceutical grade Hep is derived from mucosal tissues of slaughtered meat of animals such as porcine (pig) intestine or bovine (cow) lung. It acts as an anticoagulant, preventing the formation of clots and extension of existing clots within the blood. It is one of the oldest drugs in widespread clinical uses. Its discovery in 1916 predates the establishment of the United States Food and Drug Administration, although it did not enter clinical trials until 1935.

It was originally isolated from Canine liver cells hence its name (hepar is Greek word for "Liver") Heparin. Hep and its derivatives are effective for preventing thromboses and to cure urgent vein thrombus.² It has variety of biological activities such as anticoagulant, antilipemic, antithrombotic, immunoregulatory, antiphlogistic and antianaphylactic activities, etc.³ So Hep level in the patient's blood need to be carefully and accurately monitored during surgery and recovery. The reported methods of Hep determination focused on: Flowing injection analysis,⁴ capillary chromatography,⁵ surface plasmon resonance sensor analysis,⁶ membrane electrode *via* protamine titration⁷ and extracorporeal membrane oxygenation.⁸ The methods mentioned above have high fluorescence quantum yield, large

stokes shift, narrow emission bands and a large fluorescence lifetime. Hence, they avoid potential background fluorescent emission interference from the biological matrix.⁹ The some of researchers have been reported to detect the Hep by using rare earth fluorescent probe such as Tb,^{3+,9,10} Eu³⁺.¹¹ But there was no report on NFLX-Ce³⁺ complex as a fluorescent probe. Therefore a new method with high sensitivity and selectivity for the spectrofluorometric determination of Hep is established.

In this paper, the interaction of NFLX-Ce³⁺-Hep fluorescence system was studied. Experimental results show that the characteristics peak of Ce³⁺ at 356 nm can be greatly enhanced and the enhancement effect is shown to relate with the concentration of Hep. This method is simple relatively free of interference from coexisting substances and can be successfully applied to determine the Hep in heparin sodium injection samples with satisfactory results. By using the Rosenthal graphic method, the binding number and association constant of Hep with the probe has been estimated. The mechanism of fluorescence enhancement between NFLX-Ce³⁺ complex and NFLX-Ce³⁺-Hep were also studied.

Experimental

Reagents. All the reagents are of higher grade. The deionised, doubly distilled water was used through out the experiments. Hep was procured from s.d. fine-chem. Limited Mumbai India. A stock solution of Hep (1.0 mg · mL⁻¹) was prepared directly by dissolving appropriate amount of it in doubly distilled water. NFLX was obtained as a gift sample from pharmaceutical companies and standard solution 2.20 × 10⁻⁵ mol · L⁻¹ was prepared by dissolving in a small amount of HCl and then diluted with distilled water. The stock solutions were stored at 0 - 4 °C

Aqueous stock solution of Ce³⁺ ion 1.0 × 10⁻⁴ mol · L⁻¹ was prepared by dissolving the cerium chloride with doubly distilled water. The Tris-HCl buffer solution (0.1 mol · L⁻¹) was prepared

by dissolving 2.424 g of tris in 100 mL volumetric flask with water and adjusted the pH with HCl/NaOH.

Apparatus. All fluorescence spectra were recorded on PC based spectrofluorometer (JASCO Japan FP-750) and absorption spectra were recorded with UV-Visible-NIR spectrophotometer (Shimadzu, Model UV-3600). The pH measurements were carried out on pH meter (model LI-10T Elico Pvt. Ltd, India).

General Procedure. An aliquot of standard Ce^{3+} solution ($1.0 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$) was taken into 10 mL calibrated tubes, followed by addition of 0.5 mL $2.20 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ NFLX solutions, 1 mL of $10.0 \mu\text{g} \cdot \text{mL}^{-1}$ Hep solution and 0.5 mL buffer solution. The mixture was diluted to 10 mL with doubly distilled water and allowed to stand for 10 minutes at room temperature. The fluorescence intensity was measured in a 1 cm quartz cell at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 259/356 \text{ nm}$. The excitation and emission slit width were kept constant at 10 nm for the measurement. The enhanced fluorescence intensity of NFLX- Ce^{3+} by Hep was represented as,

$$\Delta F = F - F_0 \quad (1)$$

Here F and F_0 are the fluorescence intensities of the systems with and without Hep, respectively.

Results and Discussion

Fluorescence Spectra. Fluorescence excitation spectra and Fluorescence emission spectra of 1. NFLX, 2. Ce^{3+} , 3. Ce^{3+} -Hep, 4. NFLX-Hep, 5. Ce^{3+} -NFLX, 6. Hep and 7. NFLX-Hep- Ce^{3+} are shown in Figure 1(a) and 1(b) respectively. From the figure, it is observed that pure Ce^{3+} ion solution has a nearly no peak. In Fig. 1 comparing curve 1 with curve 5, after the addition of NFLX into the Ce^{3+} ion solution, NFLX can form a binary complex with Ce^{3+} ion and observed the characteristic spectrum of Ce^{3+} ion appears at $\lambda_{\text{ex}} = 259 \text{ nm}$ and $\lambda_{\text{em}} = 356 \text{ nm}$. The fluorescence intensities of Ce^{3+} ion at 356 nm can be enhanced remarkably after the addition of Hep into the NFLX- Ce^{3+} system, which indicates that Hep can form a very stable ternary complex with the NFLX- Ce^{3+} binary complex.

Absorption Spectra. The absorption spectra of 1. NFLX, 2. Ce^{3+} , 3. Ce^{3+} -Hep, 4. NFLX-Hep, 5. NFLX- Ce^{3+} , 6. Hep and 7. NFLX- Ce^{3+} -Hep are shown in Fig. 2. From Fig. it can be seen that curve 2, 3, and 6 all have nearly no peaks. The addition of Ce^{3+} increases the absorption but does not introduce any spectral changes in the absorption spectrum of NFLX. Similarly addition of Hep also enhance the absorption of NFLX with slight blue spectral shift. These observations rules out the possibility of formation of NFLX- Ce^{3+} and NFLX- Ce^{3+} -Hep complexes in the ground state. The excitation spectra of NFLX without Ce^{3+} is identical spectrally with its absorption spectra, and exhibited pronounced blue shift after addition of Ce^{3+} and further addition of Hep the intensity seen to enhanced. These observation led us to consider the formation of NFLX- Ce^{3+} -Hep ternary complex.

Optimum Conditions. Effect of pH: The effects of various kinds of buffers on the fluorescence intensity of the system were tested and it was found that the Tris-HCl is the best for pro-

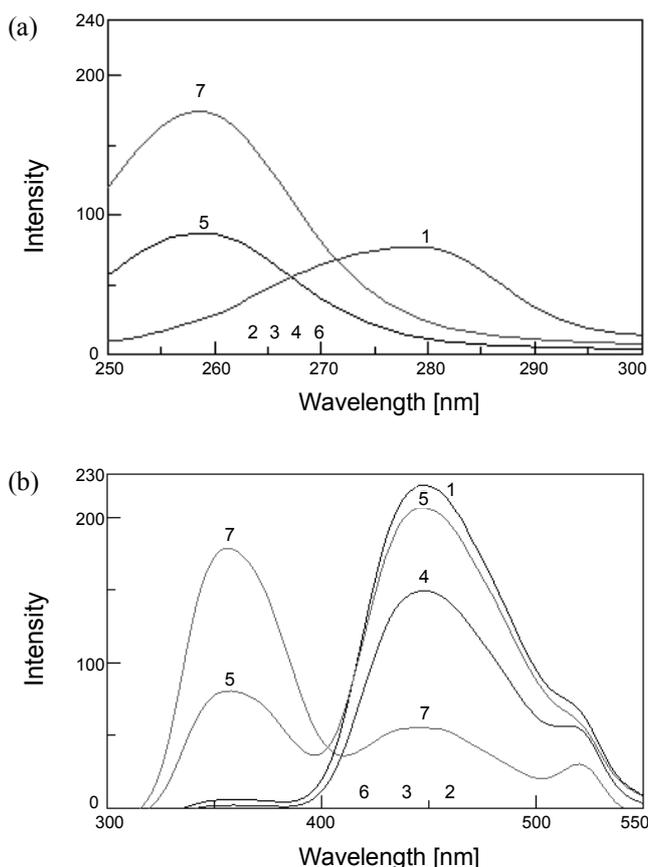


Figure 1. (a) Fluorescence Excitation spectra: 1: NFLX, 2: Ce^{3+} , 3: Ce^{3+} -Hep, 4: NFLX-Hep, 5: Ce^{3+} -NFLX, 6: Hep, 7: NFLX-Hep- Ce^{3+} . Experimental condition: NFLX: $2.20 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$, Ce^{3+} : $1.0 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$, Hep: $10.0 \mu\text{g} \cdot \text{mL}^{-1}$, Buffer pH = 7.60, $\lambda_{\text{ex}}/\lambda_{\text{em}} = 259 \text{ nm}/356 \text{ nm}$. (b) Fluorescence Emission spectra ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 259 \text{ nm}/356 \text{ nm}$). 1: NFLX, 2: Ce^{3+} , 3: Ce^{3+} -Hep, 4: NFLX-Hep, 5: Ce^{3+} -NFLX, 6: Hep, 7: NFLX-Hep- Ce^{3+} .

posed system. The effect of pH on the fluorescence intensity is shown in Fig. 3. From the figure it is observed that the fluorescence intensity was enhanced maximum in the range of pH 6.8 to 8.2. Therefore the pH 7.6 was fixed for further study.

Effect of NFLX Concentration: The effect of NFLX concentration on the fluorescence intensity of the system was studied when concentration of NFLX solution added in the range of $2.20 \times 10^{-7} \text{ mol} \cdot \text{L}^{-1}$ to $2.20 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}$ to the NFLX- Ce^{3+} -Hep system, the fluorescence intensity reached maximum and remained constant. Thus $1.1 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}$ NFLX was used for further study.

Effect of Ce^{3+} Ion Concentration: In order to study the effect of Ce^{3+} ion concentration, the fluorescence intensity measurement of the system was carried out by varying the concentration of Ce^{3+} ion from $1 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ to $2 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ (Fig. 4). The result indicates that the maximum fluorescence intensity was observed in the studied concentration range. Hence all measurements were carried out at $1.5 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ for further study.

Effect of Equilibration Time: Equilibration time for completing the chelation reaction was varied between 5 to 30 minutes at room temperature. The fluorescence intensity reached its maximum value and remained stable at least 2 hours then

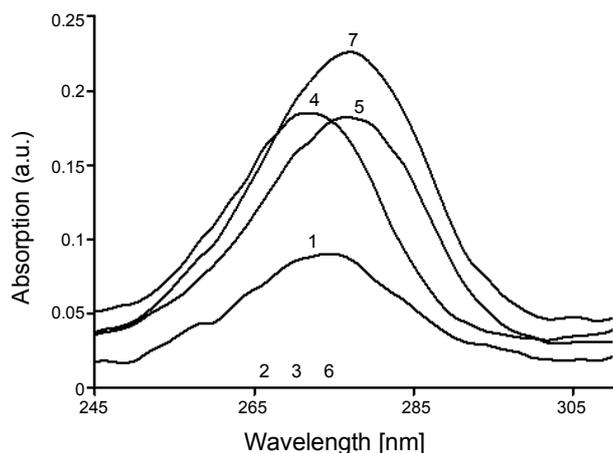


Figure 2. Absorption Spectra of (1) NFLX, (2) Ce^{3+} , (3) Ce^{3+} -Hep, (4) NFLX-Hep, (5) NFLX- Ce^{3+} , (6) Hep, (7) NFLX- Ce^{3+} -Hep. Experimental condition: NFLX: $2.20 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$, Ce^{3+} : $1.0 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$, Hep: $10.0 \mu\text{g} \cdot \text{mL}^{-1}$, Buffer pH = 7.60.

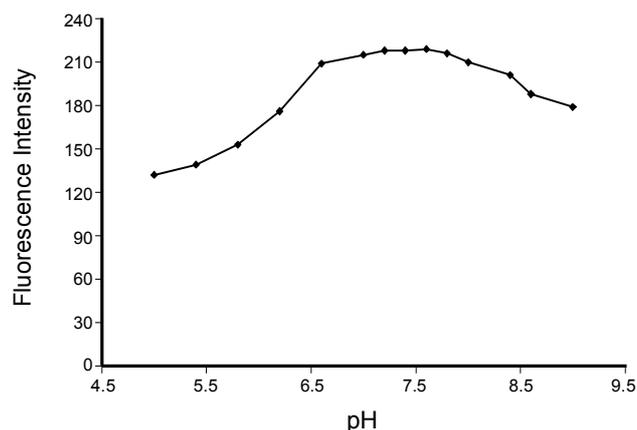


Figure 3. Effect of pH on the enhanced fluorescence intensity; The Tris-HCl buffer is used for pH 7.0 - 8.0.

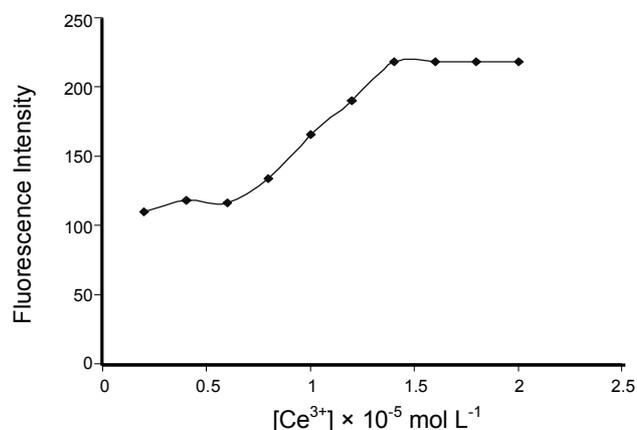


Figure 4. Effect of the concentration of Ce^{3+} on the enhanced fluorescence intensity. A 10 mL solution contains NFLX ($1.1 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}$), HEP ($1.0 \mu\text{g} \cdot \text{mL}^{-1}$), buffer solution (pH 7.60) and Ce^{3+} ($1.0 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$, $2.0 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$).

Table 1. Effect of coexisting substances

Sr. No.	Coexisting substance	Concentration (mol L^{-1})	Average Fluorescence Intensity ($\Delta\text{F}\%$)
1	$\text{CO}^{2+}(\text{Cl})$	5.0×10^{-5}	2.5
2	$\text{Cr}^{3+}(\text{SO}_4^{2-})$	1.5×10^{-6}	2.0
3	Mn^{2+}	5×10^{-5}	6.55
4	$\text{Fe}^{3+}(\text{SO}_4^{2-})$	9.0×10^{-7}	-6.1
5	Ni^{2+}	6.8×10^{-6}	-3.2
6	Sn^{4+}	8.4×10^{-7}	5.7
7	$\text{Cu}^{2+}(\text{SO}_4^{2-})$	1.6×10^{-6}	4.6
8	$\text{Zn}^{2+}(\text{Cl})$	1.1×10^{-5}	2.5
9	Tryptophan	1.5×10^{-5}	3.88
10	Al^{3+}	3.77×10^{-5}	-0.25
11	$\text{Ca}^{2+}(\text{Cl})$	5.00×10^{-5}	-2.40
12	$\text{Mg}^{2+}(\text{SO}_4^{2-})$	5.00×10^{-5}	5.10
13	HSA	2.00×10^{-7}	4.50
14	Adenine	1.04×10^{-7}	5.20
15	Glucose	1.00×10^{-3}	-2.40
16	Mo^{6+}	1.00×10^{-5}	3.69

Table 2. Comparison of spectrofluorimetric methods for the determination of Hep

Sr. No.	Method	Linear range	Detection limit
1	Flowing injection analysis	0 - 12 mg/mL	300 ng/mL
2	Capillary chromatography	0.50 - 44.1 mg/mL	157.5 ng/mL
3	Surface plasmon resonance sensor analysis	1.26 - 12.6 mg/mL	1260 ng/mL
4	Current method	0.06 - 1.5 mg/mL	60 ng/mL

decreases slowly. Hence all measurements were made at room temperature within 2 hours.

Effect of Addition Order: The addition of various reagents in different orders has a great influence on the fluorescence intensity. Therefore considering the enhancement of fluorescence intensities of these systems the order of addition were maintained as; NFLX, Ce^{3+} , Hep and buffer.

Effect of Coexisting Substances: The effect of various cations and anions (coexist substances) commonly associated with the fluorescence of NFLX- Ce^{3+} -Hep system was studied under optimum conditions. The criterion for interference was fixed at a $\pm 10\%$ variation of average fluorescence intensity calculated for the established level of Hep. The tolerance limits for the tested ions are given in Table 1 and it can be seen that the most coexisting substances have no influence.¹¹

Analytical Application

Linear Range and Limit of Detection. Limit of detection is the lowest quantity of substance that can distinguish from the absence of that substance within a stated confidence limit. Under the experimental conditions there was a linear relationship between fluorescence intensity and Hep concentration in the range of 0.1 to 1.5 $\mu\text{g}/\text{mL}$ with a correlation coefficient 0.9893.

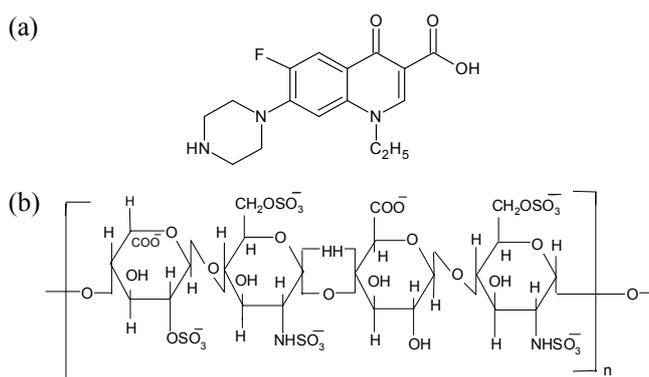


Figure 5. (a) Structure of Norfloxacin, (b) Structure of Heparin.

The limit of detection and standard deviation are 60 ng/mL and 0.287 respectively from a series of 10 reagents blanks. Experimental results are shown in Table 2. By comparison with other existing methods, it is observed that the proposed method has advantages over others considering following factors: good stability, high sensitivity and wide linear range.

Determination of Heparin in Heparin Sodium Injection Sample. The developed method was applied for the determination of Hep in Hep sodium injection sample. For the assay of Hep, the sample must be diluted appropriately within the linear range of the determination of Hep and the sample solution was analyzed by the method developed above, using the standard calibration method. The average recovery and relative standard deviation for the determination of Hep from injection sample are 104.8 (%) and 0.3 respectively. This indicates the superiority of the proposed method over the other methods.

The Interaction Mechanism of the Complex NFLX-Ce³⁺ with Heparin. The Formation of NFLX-Ce³⁺ Binary Complex: Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid] is broad spectrum antimicrobial fluoroquinolone. The antimicrobial spectrum of NFLX makes this drug attractive in veterinary therapy. NFLX reaches high levels in the urine and it is used in the treatment of urinary tract infections. The structure of NFLX is shown in Fig. 5(a).

Norfloxacin is one kind of bacteriophage containing an α -carbonyl carboxylic acid configuration. It is suitable for efficient energy transfer from ligand to Ce³⁺ ion. The high fluorescence quantum yield is observed due to large Stokes shift, narrow emission bands and a large fluorescence life time and so avoid potential background fluorescent emission interferences from biological matrix. Therefore NFLX is an ideal ligand for Ce³⁺ ion and it can possibly sensitize the fluorescence intensity of Ce³⁺ ion *via* intra molecular energy transfer¹².

The Formation of NFLX-Ce³⁺-Hep Ternary Complex: Hep is a naturally occurring biomacromolecule with an average molecular weight 15000 g/mol and an average charge -70. Hep is member of the glycosaminoglycan family of carbohydrates. It consists of repeating disaccharide units of glucuronic acid and glucosamine residues shown in Fig. 5(b).

Whole Hep molecule exists as a big polyvalent anionic state in water solution. Because Hep has 7 binding sites (five sulfate groups and two carboxyl groups) per tetrasaccharide unit, and

the used Hep contains 42 monosaccharide units, the total binding number is 73.5 (42 \times 7/4) per Hep molecule.³ Therefore a stable ternary complex in close proximity to a large degree of molecular conjugation and rigid structure can be formed by the electrostatic interaction and coordination between Hep and NFLX-Ce³⁺ binary complex.

Measurement of Association Constant and Binding Numbers: The Rosenthal graphic method¹³ was regarded as a modification of the Scatchard method. The equation for a Scatchard plot is slightly different from the equation for the Rosenthal plot. The Rosenthal graphic method was used to estimate the association constant (K) and binding number (N) of the biomacromolecule to the NFLX-Ce³⁺ probe. Briefly, when C, C_b, C_f and C_{Ce³⁺-NFLX} are constant biomacromolecule concentration in the system, the Hep bound, free and total concentration of the complex respectively. Then the Rosenthal plot shows equation (2)^{14,15}

$$\frac{C_b}{C_f} = -(C_{Ce^{3+}-NFLX} - C_f)K + NCK \quad (2)$$

$$\text{Because } C_{Ce^{3+}-NFLX} = C_b + C_f$$

then

$$\frac{C}{C_f} = -(C_{Ce^{3+}-NFLX} - C_f)K + NCK + 1 \quad (3)$$

In the system, if C_{Ce³⁺-NFLX} and C_f are within the dynamic range of the calibration graph for NFLX-Ce³⁺-Hep complex, equation (4) can be obtained

$$\frac{F_0}{F} = -\left(1 - \frac{F}{F_0}\right)C_{Ce^{3+}-NFLX}K + NCK + 1 \quad (4)$$

F, F₀ are the intensities of the system with and without Hep.

The plot of $\frac{F_0}{F}$ vs $\left(1 - \left(\frac{F}{F_0}\right)C_{Ce^{3+}-NFLX}\right)$ can be obtained. The value of K and N are 2.80 $\times 10^4$ L \cdot mol⁻¹ and 17.2.

Conclusions

The experiment indicates that Hep can enhance the fluorescence intensity of NFLX-Ce³⁺ complex. Under the optimum condition the enhanced fluorescence intensity is in proportion to the concentration of Hep in the range 0.1 to 1.5 μ g/mL. In comparison with most of the reported fluorescence probe,¹² NFLX-Ce³⁺ has higher sensitivity and wide range. The proposed method has been successfully applied for the determination of Hep from pharmaceutical samples directly. The interaction mechanism is also studied.

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