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The Investigation of Fluorescence Spectra and Fluorescence Quantum Yield of Enrofloxacin

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Abstract: In this paper, the fluorescence spectra of Enrofloxacin (ENR) in different pH conditions was studied in order to determine its structural changes due to protonation with pH changes. The ENR two-step dissociation constant is calculated and the fluorescence quantum yield under acidic conditions is measured. In the strong acidic conditions, ENR exists of H_3L^{2+} form of which maximum emission wavelength is at 450 nm. At the condition of pH 2.45 to 4.23, ENR exists of H_2L^+ form with strong and steady fluorescence. The maximum emission wavelength is still 450 nm. At the condition of pH more than 4.23, the maximum emission wavelengths are gradually blue shifted to 445 nm and the fluorescence intensity decrease with the increase of pH which shows that H_2L^+ loses one proton with the increase of pH and exists in the form of bipolar ion HL. When the pH is more than 12.28, the fluorescence intensity are weakened to nearly disappear with the increase of pH value, indicating that HL gradually loses the proton with the conversion to the anion of L^- which is weaker fluorescence. In the buffer solution of pH 3.00, with quinine sulfate as reference, the fluorescence quantum yield of ENR at excitation wavelength of 274 nm is 0.125.

Keywords: Enrofloxacin, Fluorescence Spectroscopy, Fluorescence Quantum Yield, Dissociation Constant

1. Introduction

Enrofloxacin (ENR) is a broad spectrum of fluoroquinolone (FQ) antibiotic with high physiological activity and among the most consumed quinolones on livestock in China [1, 2]. The widely used quinolones will then accumulate in the food chain, causing environment pollution and would possibly lead to undesired effects in humans [3, 4]. As quinolones can be synthesized by chemical methods, modifying the structure of these drugs can significantly improve its shortcomings. Thus, quantitative and qualitative study of quinolones has a great significance.

ENR molecules contain a carboxyl group and a piperazinyl amine group and could exist as cationic, zwitterionic, and anionic species in solution depending on pH values [5], which emission is closely related to the degree of protonation [6, 7]. Compared with NMR or IR, fluorescence spectroscopy has the advantages of fast speed and low cost of instruments. In this study, the fluorescence spectra of ENR under different pH conditions were investigated to determine the structural changes. The dissociation constants were calculated and the fluorescence quantum yields were measured by using quinine sulfate as reference solution. Chemical structure of ENR is shown as Figure 1.

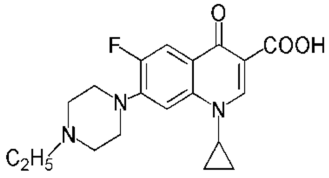


Figure 1. *Chemical structure of Enrofloxacin.*

2. Experimental

2.1. Apparatus and Materials

All fluorescence spectra were recorded using a RF-5301PC spectrofluorophotometer (Shimadzu, Japan). Absorption was measured using a UV-visible spectrophotometer (UV-265, Shimadzu, Japan). All pH measurements were carried out with a pHS-3C precision acidity meter (Leici, Shanghai, China).

ENR (biochemical reagent, Shanghai Reagent Second

Factory, China) solution (10 μM) was prepared. A series of buffer solution was configured from pure borax, potassium dihydrogen phosphate, hydrochloric acid and sodium hydroxide (biochemical reagent, Shanghai Reagent Second Factory, China). Quinine sulfate (biochemical reagent, Shanghai Reagent Second Factory, China) solution was used at a concentration of 40 μM with 0.05 M sulfuric acid solution. The reagents used in the experiments were analytical grade, and the water was double-distilled water.

The investigation was approved and that informed consent was obtained.

2.2. Procedures

ENR solution (1.0 mL; 10 μM) was added to 10 mL colorimetric tubes. The samples were diluted to a scaled volume with pH buffer solution and mixed thoroughly by shaking. The excitation and emission slits were set at 5 nm and the excitation wavelength for ENR solution was 274 nm. The fluorescence emission spectra of ENR were scanned.

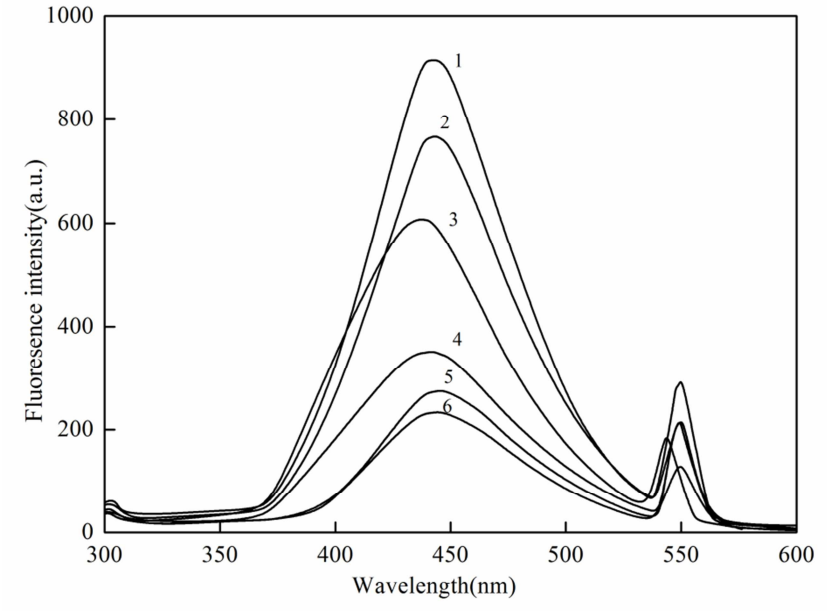
3. Results and Discussion

3.1. Fluorescence Spectra of ENR under Near-Neutral or Weakly Acidic Conditions

The fluorescence spectra of ENR under the near-neutral or weakly acid conditions are shown in Figure 2. From In the figure, the emission peak was blue shifted and decreased significantly with increasing pH. When pH was 4.23-4.94, the maximum emission wavelength was 450 nm. When pH was 5.84-7.78, the maximum emission wavelengths were blue shifted to 445 nm. This spectral characteristic indicated that the presence of ENR varies with different pH, and the fluorescence properties of these two forms are different, as shown in Figure 2.

The red shift or blue shift of the spectrum was related to the degree of conjugation of the molecule [8-10]. The phenomenon that the maximum emission wavelength was gradually blue shifted with increase in pH, indicated that ENR should have a high degree of conjugation and a more rigid structure at lower pH values. Therefore, the proton dissociation equilibrium of ENR at this pH range is shown in Figure 3.

When pH was increased to 7.78 from 4.23, ENR formed a bipolar ion HL due to the presence of H_2L^+ that loses a proton from the carboxyl group bound oxygen. Because the proton of the carboxyl group bound oxygen can be combined with the 4-carbonyl oxygen in the quinoline ring through hydrogen, the substance can form a ring structure, and then conjugated with the quinoline ring. After lossing the proton, the conjugation of the whole molecule was reduced, causing a blue shift in fluorescence emission wavelength.



(1) pH=4.23 (2) pH=4.94 (3) pH=5.84 (4) pH=6.67 (5) pH=7.14 (6) pH=7.78

Figure 2. *Fluorescence spectra of ENR under near-neutral or weakly acidic conditions.*

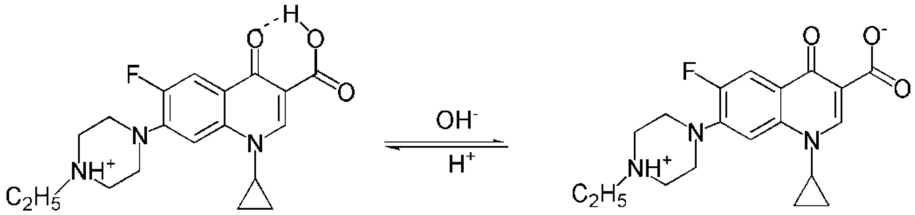
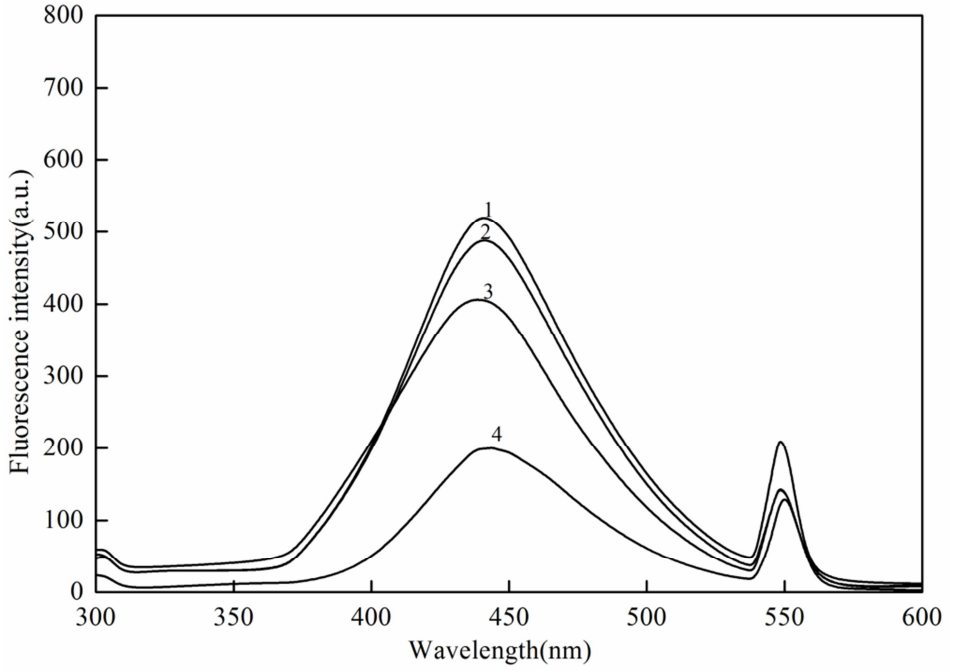


Figure 3. *The process of H_2L^+ dissociation to HL.*

3.2. Fluorescence Spectra of ENR in the Acidic Conditions

The fluorescence spectra of ENR under acidic conditions are shown in Figure 4. The emission peaks at 450 nm was increased gradually with increasing pH, and the position of maximum emission peak was basically unchanged. This result indicates that ENR may further combine with a proton under increasing acidic conditions, namely H_2L^+ to H_3L^{2+}. The binding site of the proton on ENR may be at the 1-position nitrogen in the quinoline ring, or at the carbonyl oxygen. As ENR fluorescence intensity was significantly increased under the condition as shown in Figure 4, the proton may be combined with the 1-position nitrogen in the quinoline ring within the pH range 1.02-2.81, the proton dissociation equilibrium of ENR at this pH range is shown in Figure 5. This combination can destroy the involvement of lone-pair electrons of the 1-position nitrogen in the formation of conjugated Π bonds, and significantly affects ENR fluorescence properties.



(1) pH=2.81 (2) pH=2.45 (3) pH=1.87 (4) pH=1.02.

Figure 4. *Fluorescence spectra of ENR under acidic conditions.*

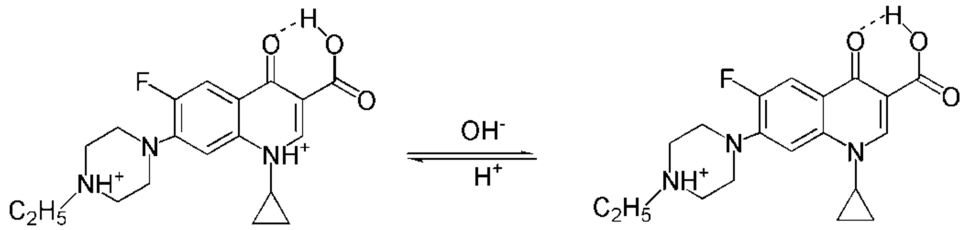
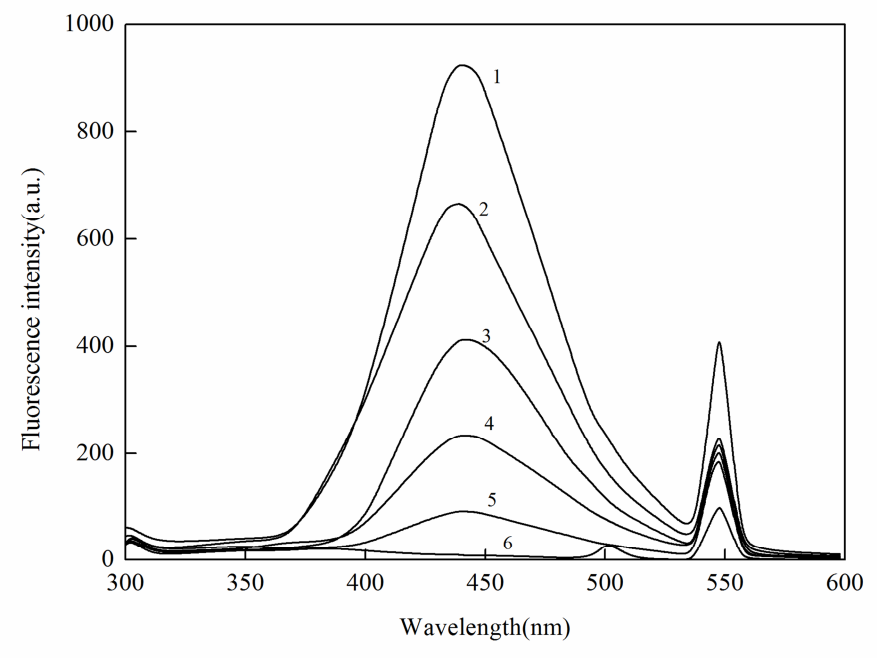


Figure 5. *The process of H_3L^{2+} dissociation to H_2L^+.*

3.3. Fluorescence Spectra of ENR in Alkaline Conditions

Figure 6 shows the fluorescence spectra of ENR under alkaline conditions. With increasing pH, the fluorescence intensity at 450 nm was reduced significantly to the extent that the fluorescence disappears, but the position of fluorescence peak was basically unchanged. This spectral characteristic indicated that the ENR bipolar ion HL gradually lost a proton, and then transformed into L^- without fluorescence. As shown in Figure 6, this transition caused a decrease in fluorescence intensity with increasing pH. The proton dissociation equilibrium of ENR at this pH range is shown in Figure 7.



(1) pH=9.65 (2) pH=10.04 (3) pH=11.05 (4) pH=11.65 (5) pH=11.95 (6) pH=12.10

Figure 6. *Fluorescence spectra of ENR under alkaline conditions.*

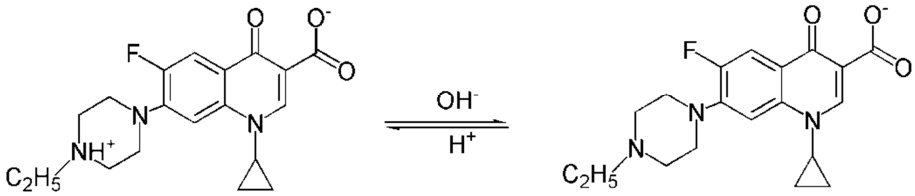


Figure 7. *The process of HL dissociated to L.*

3.4. Dissociation Constant of ENR

The dissociation constant pK_a of ENR can be calculated based on the following equation [11]:

$$pK_a = pH - \lg\left[\left(F_{HB} - F\right)\Big/\left(F - F_B\right)\right] \quad (1)$$

Where F_{HB} and F_B are the fluorescence intensities of ENR completely in the presence of a conjugated acid type or a fully conjugated base type, respectively. F is the fluorescence intensity corresponding to the pH of solution. According to the above equation, the pK_a value can be calculated as long as F_{HB}, F_B and a set of pH-F data are measured. The calculated data was shown in Table 1 and Table 2. From the data in Table 1, the pK_{a1} of ENR was 6.80 ± 0.03. From the data in Table 2, the pK_{a2} of ENR was 7.56 ± 0.10.

Table 1. *The pK_{a1} of ENR at different pH values.*

pH	F	lg[(F-F_B)/(F_{HB}-F)]	pK_{a1}	Average
5.84	925	-0.97	6.81	6.80
6.35	777	-0.46	6.81	
6.82	616	-0.01	6.83	
7.14	353	0.41	6.73	

Table 2. *The pK_{a2} of ENR at different pH values.*

pH	F	lg[(F-F_B)/(F_{HB}-F)]	pK_{a2}	Average
10.7	312	3.19	7.51	7.56
11.05	298	3.45	7.60	
11.95	270	4.25	7.70	
12.28	251	4.87	7.41	

3.5. The Fluorescence Quantum Yield of ENR Measurement

The fluorescence quantum yield is defined as the ratio of the number of photons emitted to the number of photons absorbed [12, 13]. Fluorescence intensity and the absorbance value of substance and reference substance solutions at the same excitation wavelength were measured, and fluorescence quantum yield of the determined substance was obtained based on the following equation [14, 15]:

$$Yu = Ys \cdot \left(Fu/Fs\right) \cdot \left(As/Au\right) \quad (2)$$

Where Yu and Ys are the fluorescence quantum yields of substance and reference substance, respectively. Fu and Fs are the integral fluorescence intensity of substance and reference substance, respectively. Au and As are the absorbance values of substance and reference substance at excitation

wavelengths, respectively.

The fluorescence quantum yield of ENR was measured using quinine sulfate as a reference [16-18]. The fluorescence quantum yield of quinine sulfate at the excitation wavelength 313 nm was 0.55. ENR solution (adjusted pH with buffer solution) and quinine sulfate were prepared with absorbance differences of less than 0.05. The Au and As values were recorded at a specified wavelength, then the fluorescence spectra of ENR and quinine sulfate solutions were scanned at different excitation wavelengths to calculate integrated fluorescence intensity. Finally, the fluorescence quantum yield was calculated according to Formula (2). In this experiment, concentration of ENR, $C_{(ENR)}$ =1.0 μM. The quinine sulfate concentration was 4.0 μM, and the slit width was 3 nm/ 5 nm. The results were shown in Table 3.

As shown in Table 3, the fluorescence quantum yield of quinine sulfate was basically the same as at the excitation wavelength of 270-340 nm, and the fluorescence quantum yield of ENR was almost identical as at the excitation wavelength of 250-320 nm. The fluorescence quantum yield of ENR was 0.125 at the maximum excitation wavelength of 274 nm, which indicates that ENR is a relatively strong fluorescent compound.

Table 3. *Fluorescence quantum yield of ENR and quinine sulfate.*

λ/nm	Quinine sulfate				ENR			
	F	***A***	***Y***	Y/Y_{313}	λ/nm	F_{ENR}	***A***	***Y***
270	44106	0.0099	0.49	0.89	250	18250	0.015	0.134
275	45958	0.0107	0.47	0.86	260	28364	0.021	0.123
280	56634	0.0131	0.48	0.86	270	24571	0.024	0.124
290	94145	0.0196	0.58	0.96	274	34753	0.024	0.125
300	151312	0.0303	0.55	1.00	280	35490	0.020	0.132
313	218641	0.0437	0.55	1.00	290	19732	0.011	0.131
320	222782	0.0443	0.55	1.01	300	14348	0.006	0.128
330	225356	0.0440	0.56	1.02	310	16607	0.005	0.141
340	264300	0.0524	0.55	1.01	320	17476	0.005	0.126

Integral range of quinine sulfate: 370-590 nm; integral range of ENR: 390-590 nm

4. Conclusion

The structure and fluorescence properties of ENR are closely related to the pH of the solution. When pH changes, ENR exists in a proton dissociation equilibrium:

$$H_3L^{2+} \Leftrightarrow H_2L^{+} \Leftrightarrow HL \Leftrightarrow L^{-} \quad (3)$$

Under strong acid condition, ENR exists in the H_3L^{2+} form of which maximum fluorescence emission wavelength was about 450 nm. At pH 2.45-4.23, ENR mainly exists in the H_2L^{+} form in which H_3L^{2+} loses one proton at the 1-position of nitrogen in the quinoline ring. Fluorescence intensity was strong and steady and maximum emission wavelength was at 450 nm; At pH >5.84, the maximum emission wavelengths were gradually blue shifted to 445 nm with increase in pH. ENR exists in the form of a bipolar ion HL in which H_2L^{+} loses proton from the carboxyl group. At pH > 10.04, HL transforms into the anionic L^{-} form in which HL loses one proton from the piperazine ring, leading to a decrease in fluorescence intensity. However, the position of maximum emission peak was basically unchanged. The two-step dissociation constant pK_a of ENR was calculated, the pK_{a1} was 6.80 ± 0.03 and the pK_{a2} of ENR was 7.56 ± 0.10. In buffer solution pH 3.00, with quinine sulfate as reference, the fluorescence quantum yield of ENR at the maximum excitation wavelength of 274 nm was 0.125.

Acknowledgements

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