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Spectrofluorimetric Determination of Fluoroquinolones by Fluorescence Quenching of 1,4-Dihydroxyanthraquinone

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A new, rapid, simple and sensitive method has been established based on the mechanism of fluorescence quenching for determining fluoroquinolones namely ofloxacin, norfloxacin, ciprofloxacin and gatifloxacin. The quenching was found to be very effective and appreciable. The experimental parameters affecting the fluorescence intensities were studied and optimized. Under optimal experimental conditions, the calibration curve was found to be linear between F_0/F and the concentration with the range of 0.015-3.85, 0.021-4.04, 0.018-4.26 and 0.024-4.35 μg mL^{-1} for ofloxacin, norfloxacin, ciprofloxacin and gatifloxacin, respectively. The detection limit was 0.009 μg mL^{-1} (ofloxacin), 0.012 μg mL^{-1} (norfloxacin), 0.010 μg mL^{-1} (ciprofloxacin) and 0.016 μg mL^{-1} (gatifloxacin). The mechanism of the fluorescence quenching has been discussed. The binding constant and the number of binding sites were 7.11×10^6 L mol^{-1} and 1.77 (ofloxacin), 7.05×10^5 L mol^{-1} and 1.16 (norfloxacin), 4.76×10^5 L mol^{-1} and 0.94 (ciprofloxacin), 1.23×10^6 L mol^{-1} and 1.22 (gatifloxacin), respectively. The thermodynamic parameters were obtained from data at different temperatures, $\Delta H < 0$, $\Delta S < 0$ and the mean value $\Delta G < 0$ (Table-3), the results showed that the reaction was spontaneous and the main binding force was van der Waals force. The method has been applied to the determination of pharmaceutical tablets with satisfactory results.

Key Words: Fluoroquinolones, Fluorescence quenching, 1,4-Dihydroxyanthraquinone, Binding constant, Thermodynamic parameters.

INTRODUCTION

The fluoroquinolones are a group of structurally related synthetic antibacterial agents derived from the quinolone nalidixic acid. They inhibit bacterial growth by interfering with the bacterial enzyme DNA gyrase and have been widely used in both veterinary and human medicine because of their broad antibacterial spectrum, strong antibacterial activity and good bioavailability[1-3].

Currently, the methods reported for determination of the fluoroquinolones include spectrophotometry[4,5], spectrouorometry[6-8], chromatography[9-11], capillary electrophoresis[12-14], chemiluminescence[15-17], polarography [18,19] and electrochemical analysis[20-22]. Most of the analytical methods employed for the determination of fluoroquinolones in biological fluids and pharmaceutical dosage forms are HPLC methods which showed the superiorities of high selective and sensitive[23-26]. Fluorescence spectrometry, its main advantage over HPLC method is its rapidity, the method has already been described for determination of fluoroquinolones by complex formation with small molecules[27-29], biological macromolecules[30-32], metal ions[33-35], *etc*. But the reports about fluorescence quenching between fluoroquinolones and small molecules is few[36,37] and the research on determination of fluoroquinolones by fluorescence quenching of 1,4-dihydroxyanthraquinone (1,4-DHAQ) (also called quinizarin) hasn't been reported so far.

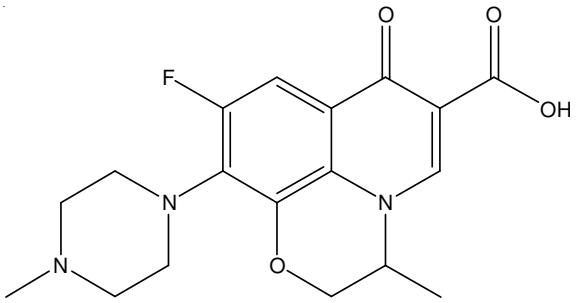
The aim of our work is to develop a rapid, simple, highly sensitive and low-cost method which can be commonly used in the determination of fluoroquinolones in pure form and pharmaceutical dosage forms. Here we report a new study on determination of four fluoroquinolones *e.g.*, ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP) and gatifloxacin (GAT) by fluorescence quenching of 1,4-DHAQ. To the reagent 1,4-DHAQ, fluoroquinolones are acted as quenchers. Compared with the method reported, this method has the following advantages: 1,4-DHAQ, the chemical reagent for the determination of fluoroquinolones, are cheap and easy to get. In addition, the reaction conditions between 1,4-DHAQ and fluoroquinolones are simple and mild. More importantly, the detection limit for fluoroquinolones is low to 0.009 μg mL^{-1}, it decreased compared to the reports[38,39], which indicated that the fluorescence quenching of 1,4-DHAQ exhibited high sensitivities for the determination of fluoroquinolones.

EXPERIMENTAL

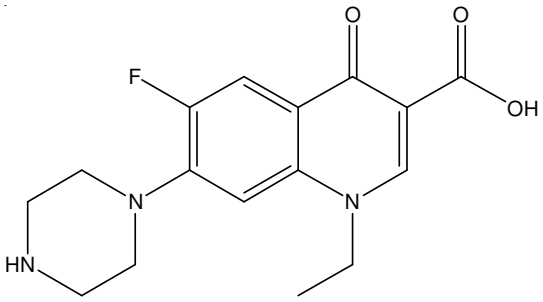
Fluorescence measurements were performed on Shimadzu RF-5301 PC spectrouorimeter (Kyoto, Japan) equipped with a hydrogen discharge lamp and 1.0 cm quartz cell. The slit

width of both excitation and emission monochromators was set at 5 nm and scan rate was 250 nm min^{-1}. The chromatographic studies were performed on an Agilent (Palo Alto, CA, USA) Mod. 1100 LC instrument equipped with degasser, quaternary pump, autosampler, column heater, containing a 20 µL loop, fast-scanning uorimetric detector. An analytical column Agilent TC-C_{18} (5 µm, 4.6 mm×150 mm) was used. An intelligent digital display temperature control water-bath Model XMTB (Yuyao, China) was used to control the temperature. A pH meter pHS-3C (Shanghai Leici Instruments Factory, China) was used for pH adjustment.

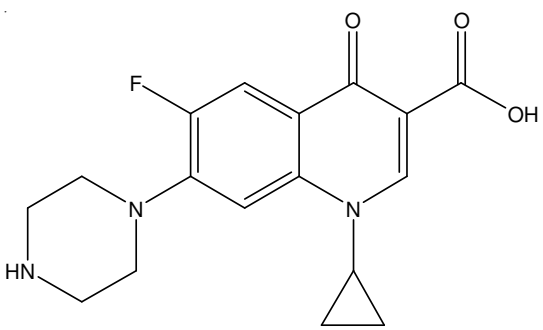
OFL, NOR, CIP and GAT were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and were used without further purification. 1,4-DHAQ was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). The chemical structures were shown in Fig. 1. All the other reagents used were of analytical reagent grade and the solutions were prepared with double distilled water.



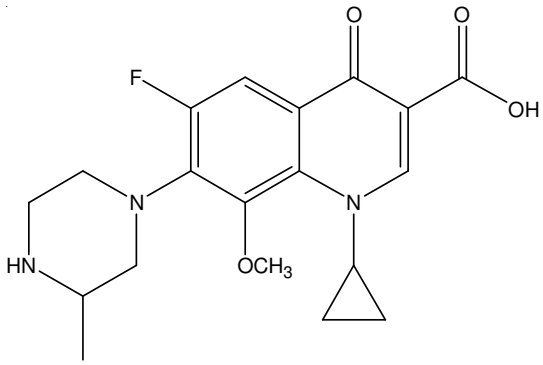
Ofloxacin (OFL)



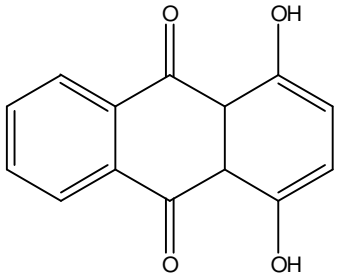
Norflocaxin (NOR)



Ciprofloxacin (CIP)



Gatifloxacin (GAT)



1,4-Dihydroxyanthraquinone (1,4-DHAQ)

Fig. 1. Structures of four fluoroquinolones and 1,4-DHAQ evaluated in this study

The stock solutions of fluoroquinolones (100 µg mL^{-1}) were prepared in 1.0 mM HCl. The stock solution of 1,4-DHAQ (500 µg mL^{-1}) was prepared in methanol. Solutions of fluoroquinolones and 1,4-DHAQ were all stored at 277.15 K in the dark, the working solutions were obtained by appropriate dilution of the stock solution with the phosphate buffer (50 mM, pH 7.0).

The mobile phase for HPLC consisted of 10 mM tetrabutylammonium bromide (pH adjusted to 3.0 with phosphoric acid) and acetonitrile (85:15, v/v), using a flow rate of 1 mL min^{-1}.

General procedure: 0.2 mL OFL (20 µgmL^{-1}), 0.2 mL 1,4-DHAQ (500 µg mL^{-1}) and 1.0 mL phosphate buffer solution (pH 7.0) were transferred sequentially to a 10.0 mL volumetric flask, the solution was diluted to the mark with double distilled water and kept heating in water bath at 323 K for 35 min, fluorescence spectra were recorded at the temperature of 298 K.

Preparation of sample tablet solutions: Twenty tablets were weighed, then finely powdered and the portions equivalent to 100 mg fluoroquinolones were transferred into a 100 mL volumetric flask, 60 mL 1.0 mM HCl was added, shake the volumetric flask to fully dissolve, the solution was diluted to the mark with 1.0 mM HCl, mixed well and centrifuged, the supernatant was used to prepare solutions of 1.0 mg mL^{-1} of fluoroquinolones. The working sample solution obtained by dilution of supernatant was used to set up the concentrations in the range of calibration studies.

RESULTS AND DISCUSSION

Fluorescence spectra: The molecular interactions between electron acceptors and electron donors are generally associated

with the formation of charge transfer complexes. 1,4-DHAQ molecule is a π acceptor, the OFL, NOR, CIP and GAT are nitrogenous compounds as π donors, 1,4-DHAQ could form n-π complexes with these drugs (Fig. 2).

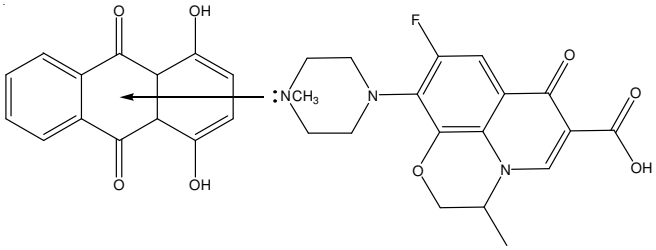


Fig. 2. Reaction between OFL and 1,4-DHAQ

These four fluoroquinolones have certain fluorescence intensities, their maximum excitation wavelength (λ^1_{ex}) and maximum emission wavelength (λ^1_{em}) are 290/494 nm (OFL), 277/444 nm (NOR), 278/449 nm (CIP), 286/484 nm (GAT), respectively. The 1,4-DHAQ has strong fluorescence intensity, its maximum excitation wavelength (λ^2_{ex}) is 479 nm and maximum emission wavelength (λ^2_{em}) is 535 nm and 560 nm. The fluorescence emission spectra of 1,4-DHAQ with the addition of OFL, NOR, CIP and GAT were shown in Fig. 3 (A) → (D), respectively. The fluorescence intensity of 1,4-DHAQ decreased remarkably with the increasing concentration of four fluoroquinolones and is directly proportional to the concentration of fluoroquinolones in a certain range. The λ_{ex} was 484 nm and the λ_{em} were at 543 nm and 568 nm for complex formed between fluoroquinolones and 1,4-DHAQ. There was significant Ex red shift 5 nm and Em red shift 8 nm with the addition of fluoroquinolones indicating that the interaction between fluoroquinolones and 1,4-DHAQ occurred and the complex may be formed.

Optimization of variables and determination

Reaction temperature study: The effect of temperature on the formation of complex for fluoroquinolones with 1,4-DHAQ was studied in the range of 298-363 K. The formed complexes were stable up to 323 K, at temperatures higher than 333 K, the fluorescence intensity increased due to dissociation of the complex at higher temperatures. Similarly, the fluorescence intensity of the complex depended on temperature is almost constant in the range of 298-323 K. Therefore, the determination of complexes was carried out at 298 ± 0.5 K.

pH study: The effect of pH on the fluorescence intensities of four reaction systems was studied. The results showed that the fluorescence intensities were almost remained constant in the pH range between 4.5 and 7.5 and decreased significantly with the change of pH below 3.5 or above 8.5. It was found that the fluorescence intensities of fluoroquinolones and 1,4-DHAQ decreased, respectively on the condition that the solution is too acid or too alkaline. The molecular structure of them is instable in extreme pH solution. Therefore, a pH of 7 was chosen with the use of phosphate buffer solution.

Reaction time study: The influence of reaction time on the formation of complex was studied. Experiments proved that after fluoroquinolones was added into the solution of 1,4-DHAQ, fluorescence intensities gradually decreased with the

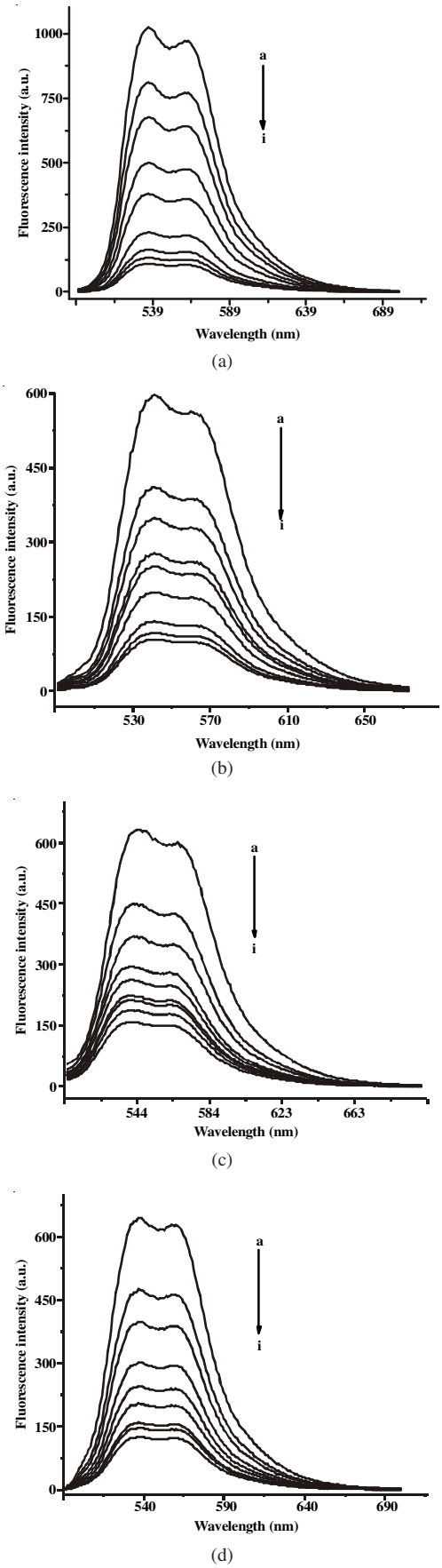


Fig. 3. Quenching effect of OFL (A), NOR (B), CIP (C) and GAT (D) on 1,4-DHAQ. λ_{ex} = 484 nm, A: 20 mg L^{-1} of 1,4-DHAQ, B, C and D: 10 mg L^{-1} of 1,4-DHAQ. a → i: 0.0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2 μg mL^{-1} OFL (A), NOR (B), CIP (C) and GAT (D)

increasing time within 30 min at 323 K and reached minimum for about 35 min, this is due to the full complex formation between fluoroquinolones and 1,4-DHAQ. Fluorescence intensities were stable at least 2.5 h at room temperature. Therefore 35 min was selected as the reaction time.

Amount of fluoroquinolones: When the concentration of 1,4-DHAQ is 10 μg mL^{-1} (5 mL), the fluorescence intensities were measured under different volumes of fluoroquinolones. The fluorescence intensity was minimum when the addition of the fluoroquinolones was about 3 mL (20 μg mL^{-1}). Thus, 1,4-DHAQ was reacted completely with fluoroquinolones when the mass ratio of fluoroquinolones and 1,4-DHAQ is 6:5.

Linear equation, detection limit and reproducibility: Under the experimental conditions described, standard calibration curves of the complexes for OFL, NOR, CIP and GAT with 1,4-DHAQ were constructed by plotting fluorescence intensities *versus* concentration. The linear regression equations and detection limit for each drug with 1,4-DHAQ are listed in Table-1. The correlation coefficients ranged from 0.9974 to 0.9993, indicating good linearity. A standard solution of fluoroquinolones (0.50 μg mL^{-1}) with 1,4-DHAQ was measured for 11 times, the mean value was 0.48 μg mL^{-1} with a RSD of 0.53 %.

Analysis of pharmaceutical samples and recovery: The sample was diluted to different concentration with double distilled water according to the range of the linear regression equation. The fluorescence intensities of solutions with different concentration were measured by the fluorescence quenching method and the HPLC method. The results of analysis of samples were shown in Table-2. Then, the recovery experiment of fluoroquinolones was tested. In addition, the four sets of data in Table-2 were tested with the Cochran test at a confidence level (p) equal to 95 %, there was no significant difference between the two methods.

Fluorescence quenching mechanism: The fluorescence quenching can occur in two major different mechanisms, static quenching and dynamic quenching. The two types of mechanism can be distinguished from each other by their different dependence on temperature and excited-state lifetime[40]. During dynamic quenching the fluorescence substance collides with quencher leading to a decrease in the quantum yield and the strength of the fluorescence. The static quenching is initiated from the formation of non-fluorescent complex between quencher and fluorophore[41].

In many cases, the fluorophore can be quenched both by collision and by complex formation with the same quencher, both static and dynamic processes can be described by the well-known Stern-Volmer equation, the Stern-Volmer plot exhibits an upward curvature. The polynomial equation is as follows:

$$\frac{F_0}{F} = (1 + K_D[Q]) + (1 + K_S[Q]$$

$$= 1 + (K_D + K_S)[Q] + K_D K_S[Q]^2 \quad (1)$$

where F_0 and F denote the fluorescence intensities in the absence and in the presence of quencher, respectively. K_D and K_S are the dynamic and static quenching constants. [Q] is the concentration of quencher. The four fluoroquinolones were used as quenchers in this experiment. Fig. 4 shows the Stern-Volmer plots for the 1,4-DHAQ fluorescence quenching by OFL (a), NOR (b), CIP (c) and GAT (d). The result indicated that the quenching mechanism between fluoroquinolones and 1,4-DHAQ was not the combined static and dynamic quenching because the Stern-Volmer plot was linear.

The completely dynamic quenching can be expected by the classical Stern-Volmer relationship[42].

$$K_{sv} = K_q \tau_0 \quad (2)$$

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{sv}[Q] \quad (3)$$

where K_q is the bimolecular quenching rate constant in M^{-1} S^{-1}, τ_0 is the lifetime of the fluorophore in the absence of quencher and K_{sv} is the Stern-Volmer quenching constant in M^{-1}. In this case, a linear plot of F_0/F against [Q] will be obtained.

In the case of static quenching, the Stern-Volmer equation is observed, giving a decrease of fluorescence intensity due to the formation of non-fluorescent complex.

$$\frac{F_0}{F} = 1 + K[Q] \quad (4)$$

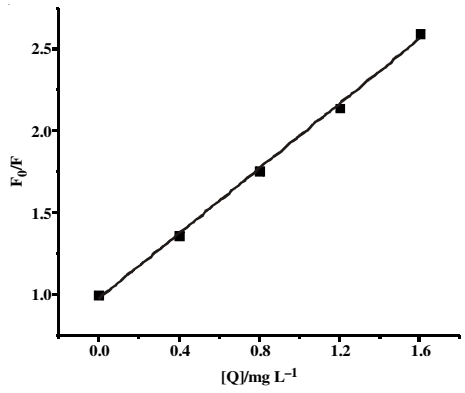
where K is the formation constant.

TABLE-1
CHARACTERISTIC PARAMETERS FOR COMPLEXES OF FOUR FLUOROQUINOLONES WITH 1,4-DHAQ

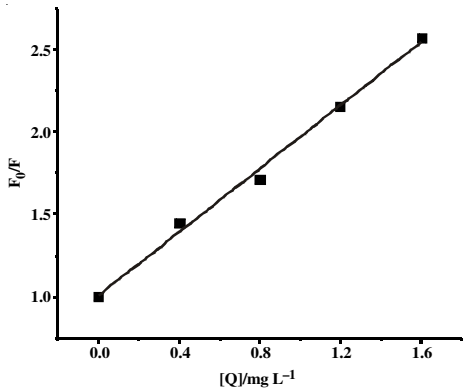
Parameters	OFL-1,4-DHAQ	NOR-1,4-DHAQ	CIP-1,4-DHAQ	GAT-1,4-DHAQ
Linear equation	$y=6.23\times10^3x-11.0$	$y=6.84\times10^3x-11.4$	$y=6.34\times10^3x-9.27$	$y=6.29\times10^3x-10.7$
Correlation coefficients (r)	0.9985	0.9980	0.9993	0.9974
Linear range (μgmL^{-1})	0.015-3.85	0.021-4.04	0.018-4.26	0.024-4.35
Limit of detection (μgmL^{-1})	0.009	0.012	0.010	0.016
Limit of quantitation (μgmL^{-1})	0.03	0.039	0.036	0.052
Number of points (n)	1.77	1.16	0.94	1.22

TABLE-2
ANALYSIS OF SAMPLES AND RECOVERY OF FLUOROQUINOLONES
(THE RECOVERY EXPERIMENT WAS TESTED BY THE PROPOSED METHOD)

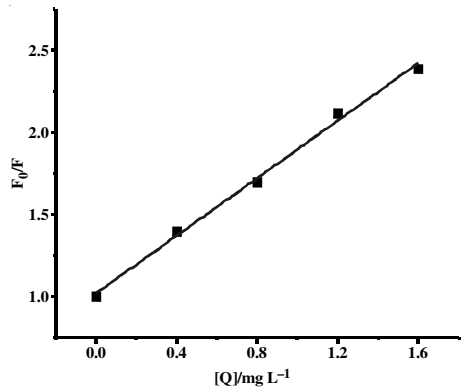
Sample No.	Proposed method (μg mL^{-1})	HPLC method (μg mL^{-1})	Fluoroquinolone (μgmL^{-1})	Average recovery (%)		RSD (%)
				Added	Found	
1	0.27	0.31	0.50	0.71	97.4	0.67
2	0.52	0.59	0.50	1.08	101.3	1.19
3	0.83	0.92	0.50	1.25	98.6	0.79
4	1.05	1.22	0.50	1.36	95.9	0.64



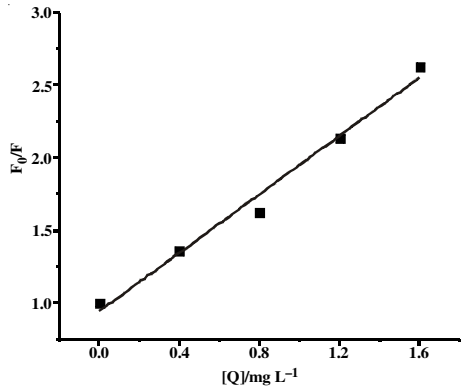
(a)



(b)



(c)



(d)

Fig. 4. Linear Stern-Volmer plots of fluorescence quenching of 1,4-DHAQ by OFL (a), NOR (b), CIP (c), and GAT (d) at 298 K

The measurement of fluorescence lifetime can conform a dynamic or static quenching process. The lifetime of fluorescence molecule on excited state has no change in the presence of quencher if static quenching takes place. Reversely, τ_0 has to be shorter if dynamic quenching occurs. That is, $\tau_0/\tau_1 = 1$ (τ_1 is the fluorescence lifetimes of fluorescence molecule in the presence of quencher) for static quenching; $\tau_0/\tau_1 = F_0/F$ for dynamic quenching. The fluorescence lifetimes of 1,4-DHAQ in the absence and in the presence of fluoroquinolones, τ_0 and τ_1, were 4.56 and 4.43 ns, respectively. $\tau_0/\tau_1 \cong 1$. Therefore, we suggested that a static quenching process was occurring between fluoroquinolones and 1,4-DHAQ.

Formation constant and the number of binding sites: For the static quenching interaction, if there are some similar and independent binding numbers in the fluorescence molecule, the following formula can be concluded between the fluorescence molecule and quencher:

$$nQ + B \rightarrow Q_nB \tag{5}$$

$$K_a = \frac{[Q_nB]}{[Q]^n[B]} \tag{6}$$

where B is the fluorescence molecule, Q is the quenchable drug molecule, n is the number of binding sites per fluorescence molecule, Q_nB is the complex molecules, which has no or weak fluorescence, K_a is the formation constant of the reaction.

$$[B_0] = [Q_nB] + [B] \tag{7}$$

here $[B_0]$ is the total concentration of fluorescence molecule including bound and unbound with the quenchable drug molecule, [B] is the concentration of unbound fluorescent molecule.

For static quenching, the fluorescence intensity is proportional to the concentration of unbound fluorescent molecule. The relationship between them is $[B]/[B_0] = F/F_0$, so there is the following equation:

$$\log\left[\frac{(F_0 - F)}{F}\right] = \log K_a + n \log [Q] \tag{8}$$

where n was the slope and log K was the intercept. The results were as shown in Fig. 5, the formation constant $K = 7.11 \times 10^6$ L mol^{-1} (OFL), $K = 7.05 \times 10^5$ L mol^{-1} (NOR), $K = 4.76 \times 10^5$ L mol^{-1} (CIP), $K = 1.23 \times 10^6$ L mol^{-1} (GAT), respectively. The number of binding sites n = 1.77 (OFL), n = 1.16 (NOR), n = 0.94 (CIP), n = 1.22 (GAT) were obtained.

Thermodynamic parameters and binding model: The molecular interaction forces between 1,4-DHAQ and fluoroquinolones may comprise van der Waals, ionic, hydrophobic, hydrogen bonds and electrostatic interactions, *etc*. The thermodynamic parameters of binding reaction could be used as the main evidence for confirming these acting forces. In order to elucidate the interaction between fluoroquinolones and 1,4-DHAQ, the thermodynamic parameters were calculated from the Van't Hoff equation:

$$\ln K = \frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{9}$$

where K is the binding constant at the corresponding temperature and R is the gas constant. The temperatures used are 298, 303, 308, 313 and 318 K. Gibbs free energy change (ΔG) is calculated from the following equation:

TABLE-3
THERMODYNAMIC PARAMETERS OF INTERACTIONS BETWEEN FLUOROQUINOLONES AND 1,4-DHAQ

Compounds	Linear equations	r	ΔH (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})	ΔG (298 K,J Kmol^{-1})
OFL	$y = 2.04 \times 10^4 x - 52.86$	0.9983	-54.98	-164.67	-5.94
NOR	$y = 1.78 \times 10^4 x - 57.69$	0.9991	-52.09	-137.39	-11.15
CIP	$y = 2.39 \times 10^4 x - 49.23$	0.9969	-51.97	-128.35	-13.72
GAT	$y = 2.39 \times 10^4 x - 47.39$	0.9975	-58.73	-119.38	-23.15

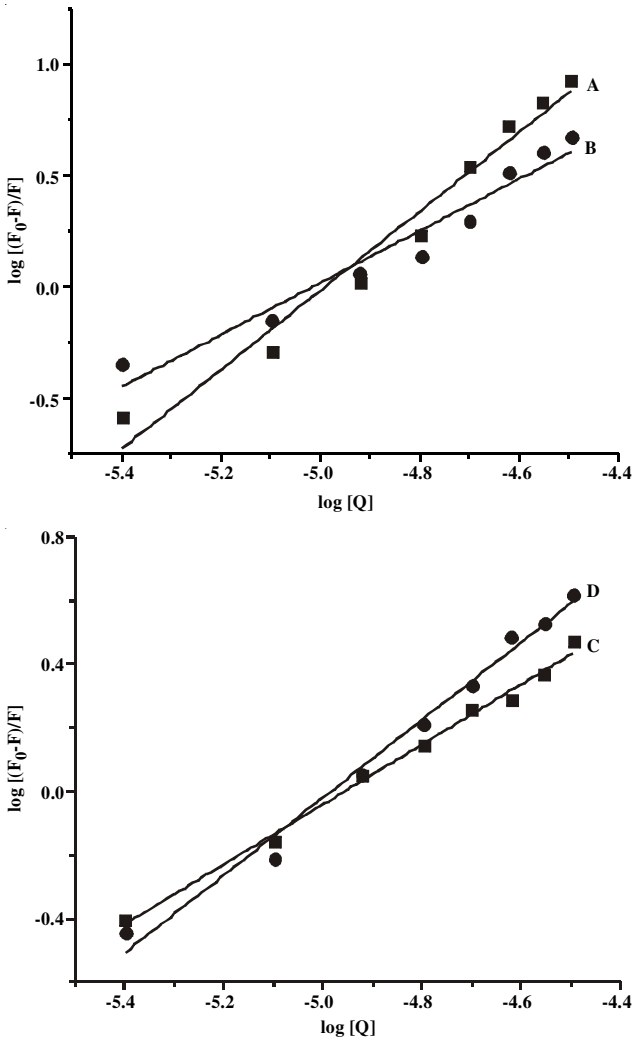


Fig. 5. Double-log plots of OFL (A), NOR (B), CIP (C) and GAT (D) quenching effects on 1,4-DHAQ at 298 K

$$\Delta G = \Delta H - T\Delta S \quad (10)$$

Enthalpy change (ΔH) and entropy change (ΔS) were calculated from the slope and intercept of the eqn. 9. ΔG were obtained according to the eqn. 10. The thermodynamic parameters calculated were shown in Fig. 6 and Table-3.

The results showed that ΔG < 0, ΔS < 0 and ΔH < 0, the negative values of ΔG revealed that the interaction process was spontaneous. What is more, we conclude that van der Waals interaction plays an important role in the binding processes of these fluoroquinolones to 1,4-DHAQ because both ΔS and ΔH are negative.

Conclusion

The fluorescence quenching mechanism between fluoroquinolones and 1,4-DHAQ was studied. The reaction was spontaneous and exothermic, the binding constant and

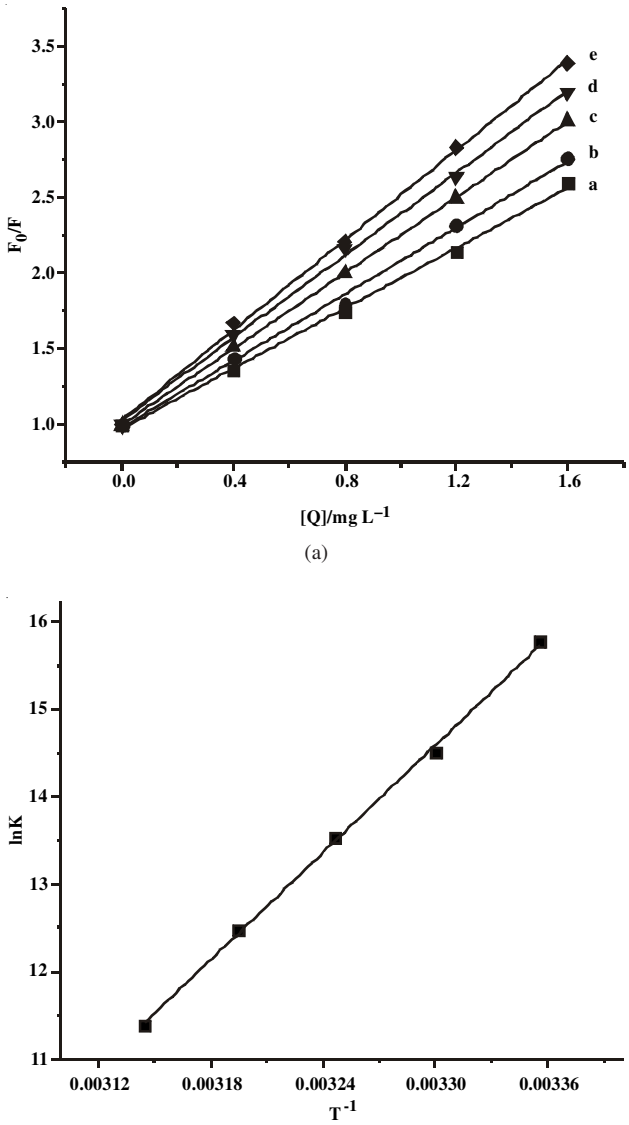


Fig. 6. (A) The Stern-Volmer plot for the binding of 1,4-DHAQ with OFL at 298 K (a), 303 K (b), 308 K (c), 313 K (d) and 318 K (e). (B) Van't Hoff plot for the binding of 1,4-DHAQ with OFL

the number of binding sites were obtained, respectively. The main binding force was van der Waals force because both ΔH and ΔS were negative. In addition, a new method for determination fluoroquinolones has also been established based on the mechanism of fluorescence quenching.

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