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Absorption Study of Norfloxacin – DNA Interaction

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Summary: Norfloxacin, a quinolone antibacterial reagent, has been studied with respect to its binding to calf thymus DNA using absorption spectroscopy. We examined the self-association of the norfloxacin, in order to determine the molar absorption coefficient of the monomer, the molar absorption coefficient of the dimer and the dimerization constant. We also examined the interaction of norfloxacin with DNA by measuring the number of binding sites per DNA segment and the binding constant. Hypochromism, broadening of the envelope and a red-shift in the drug absorption region are apparent for the norfloxacin that bound to DNA. The results were rationalized taking into account both self-association of the drug and the cooperatively effects, in terms of several literature models, Benesi-Hildebrand, Scott, Scatchard, Schwarz and Watanabe-Schwarz.

Keywords: absorption spectroscopy; DNA; norfloxacin; quinolone

Introduction

The term quinolones is used for the quinolonecarboxylic acids or 4-quinolones, which are a group of synthetic antibacterial agents containing a 4-oxo-1,4-dihydroquinoline skeleton. Since the introduction of nalidixic acid (Figure 1) into clinical practice in the early 1960s, a number of structurally related highly potent broad-spectrum antibacterial agents has been isolated.[1]

Modifications of nalidixic acid were made based on structure – activity relationships. It was discovered that a fluorine atom at position 6 and a piperazine ring at position 7 greatly enhance the spectrum of activity. The fluoroquinolones are very active against aerobic Gram-negative microorganisms but less active against aerobic Gram-positive microorganisms.[2] They are useful for the treatment of a variety of infections, including urinary tract infections, respiratory infections, sexually transmitted diseases, acute bronchitis and sinusitis.[1,2]

Fluoroquinolones develop its pharmacological action via specific inhibition of sub-unit A of the bacterial gyrase, enzyme that controls DNA shape. The mechanism of this action is unclear but these drugs interact directly with DNA in synergy with gyrase enzyme.[3] Contributions to deeper insight into the mechanism of interaction of this class of antibiotics with DNA would be important for the understanding of their therapeutic efficacy.

Norfloxacin [1-ethyl-6-fluoro-1,4–dihydro-4-oxo-7-(1-piperazinyl) quinoline-3-carboxylic acid] is a synthetic fluoroquinolone antibacterial agent. It has been used for a number of years effectively in humans and animals in the treatments of several bacterial infections, such as *Escherichia coli, Staphylococcus aureus*, *Citrobacter freundi*, *Pseudomonas aeruginosa* and *Shigella*.[4] The structure of norfloxacin is shown in Figure 2.

The interaction of norfloxacin with calf thymus DNA has studied by absorption spectroscopy. The study being points to following issues:

- **the self-association of drug**, in order to determine of the molar absorption coefficient of the monomer (ε_M), the molar

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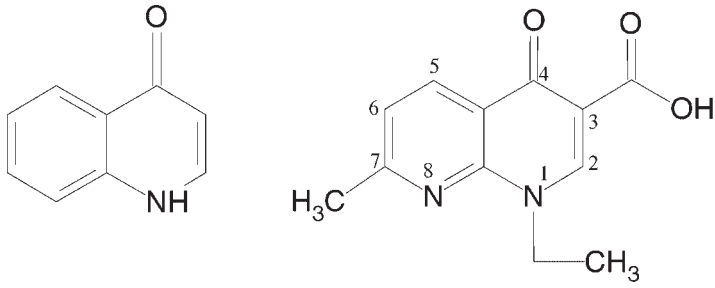

Figure 1.
The formulas of 4-oxo-1,4-dihydroquinoline (left) and nalidixic acid (right).

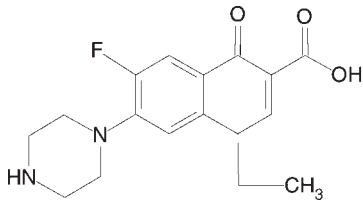

Figure 2.
The formula of norfloxacin.

absorption coefficient of the dimer (ε_D) and the dimerization constant (K_d),

- **the interaction of drug with DNA**, in order to determine of the molar absorption coefficient of stacked drug molecules on the polymer (ε_{st}), the number of binding sites per DNA segment (n) and the binding constant (K).

The results of norfloxacin - DNA system were rationalized taking into account both self-association of the drug and the cooperatively effects, in terms of several literature models, Benesi-Hildebrand, Scott, Scatchard, Schwarz and Watanabe-Schwarz.

Experimental Part

Calf thymus DNA and norfloxacin were obtained from Sigma-Aldrich, USA. The stock solutions of DNA and norfloxacin were prepared by dissolving commercially purchased reagents in doubly distilled water. The concentrations of the stock solutions of reagents were determined by the molar absorption coefficients: $\varepsilon_{260nm}=6600M^{-1}cm^{-1}$ for DNA[5] and $\varepsilon_{275nm}=37500M^{-1}cm^{-1}$ for norfloxacin.[6] The absorption measurements were performed on a Perkin-Elmer Lambda 25 UV-VIS spectrophotometer using the 1cm optical path length quartz cell, at room temperature.

Results and Discussion

In Figure 3 there is presented the absorption spectra of norfloxacin.

These spectra show a major band centred on 275 nm and two minor bands centred around 320 and 340 nm. The evolution of these spectra with the increase of drug concentration attests the formation of molecular aggregates, by analogy with other compounds[5,7] and allows the following assignment of the bands: the band at 275 nm – monomer and the bands at 320 and 340 nm – higher aggregates.

Although self-association of the drug is more adequately interpreted in terms of indefinite association models,[8] we assumed that in the range of concentrations used in this work, the presence of higher aggregates may be neglected and only the equilibrium between monomer and dimer considered. The literature describes several computation methods for estimating the dimerization constant. Applying two methods, Tipping[9] and Schwarz,[10] we have obtained a value of $\sim 240M^{-1}$ for the dimerization constant.

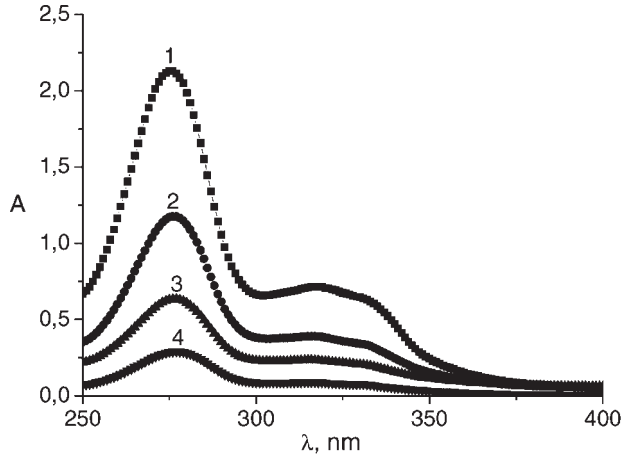

Figure 3.
Absorption spectra of norfloxacin, at different concentrations of drug: (1) $5.59 \cdot 10^{-5}$M, (2) $3.03 \cdot 10^{-5}$M, (3) $1.63 \cdot 10^{-5}$M, (4) $0.73 \cdot 10^{-5}$M.

A family of curves obtained at the titration of norfloxacin solutions of concentrations in the range 10^{-6}–10^{-5}M with calf thymus DNA is presented in Figure 4.

A marked hypochromism (up to ~15%) was observed in the 275 nm and 320 to 340 nm bands for norfloxacin - DNA complex. The interaction of the drug with calf thymus DNA causes a weak red-shift of 2nm for the maximum at 275nm, owing to the perturbation of the complexed chromophore upon binding to DNA bases, while no shift was observed for bands between 320 and 340 nm. The isosbestic point observed in norfloxacin - DNA system, implying the homogeneous conformation of the norfloxacin molecule bound to calf thymus DNA, *i.e.*, the system consists only the DNA free and DNA bound norfloxacin.

The binding constant, for 1:1 norfloxacin - DNA complex can be represented by the following equilibrium:

$$\text{Norfloxacin} + \text{DNA} \overset{K}{\rightleftharpoons} \text{Complex}$$

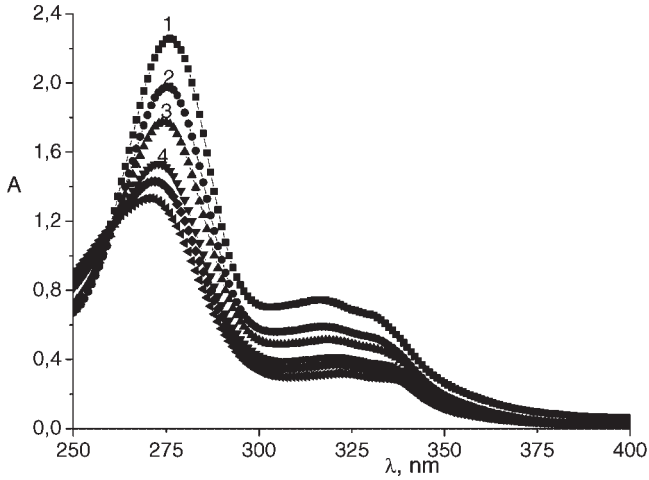

Figure 4.
Absorption spectra of norfloxacin - DNA system, at different polymer/drug ($\frac{P}{D}$) ratios: (1) 0; (2) 0.38; (3) 0.85; (4) 1.54; (5) 2.16; (6) 2.99.

Table 1.
Results of the binding constants for norfloxacin - DNA system.

Method	Equations	K, M^{-1}
Benesi-Hildebrand	$\frac{l}{\Delta A} = \frac{1}{C_D^0 \cdot K \cdot \Delta\varepsilon} \cdot \frac{1}{C_{DNA}} + \frac{1}{C_D^0 \cdot \Delta\varepsilon}$	$1{,}16 \cdot 10^4$
Scott	$\frac{l \cdot C_{DNA}}{\Delta A} = \frac{1}{C_D^0 \cdot \Delta\varepsilon} \cdot C_{DNA} + \frac{1}{C_D^0 \cdot K \cdot \Delta\varepsilon}$	$1{,}06 \cdot 10^4$
Scatchard	$\frac{\Delta A}{l \cdot C_{DNA}} = -\frac{K}{l} \cdot \Delta A + C_D^0 \cdot K \cdot \Delta\varepsilon$	$0{,}89 \cdot 10^4$
	$\frac{r}{C_F} = (n - r) \cdot K$	$0{,}94 \cdot 10^4$
Schwarz	$K = (\gamma_D^0 \cdot c_D^0)^{-1} + 2K_d$	$3{,}71 \cdot 10^4$
Watanabe-Schwarz	$\frac{2\theta - 1}{\sqrt{\theta(1-\vartheta)}} = \sqrt{\frac{q}{n}}(K \cdot C_D^0 - 1)$	$4{,}52 \cdot 10^4$

The equilibrium constant of the complex formation (K) may be estimated from the changes in the absorbance at 275 nm, using different methods, Benesi-Hildebrand,[11] Scott,[12] Scatchard,[13] Schwarz[14,15] and Watanabe-Schwarz.[16] The equations utilized and the results obtained for norfloxacin - DNA system are summarized in Table 1.

For the interaction of norfloxacin with calf thymus DNA, linear Benesi-Hildebrand, Scott and Scatchard plots were obtained; an example is given in Figure 5.

Considering the simple norfloxacin - DNA equilibrium, the absorbance is assumed to be the sum of the absorbance of the free and bound species, weighted by their respective concentrations:

$$A = f_0 \cdot (C_D^0 - C_B) + f_B \cdot C_B$$

$$A_0 = f_F \cdot C_D^0$$

where A_0 and A are the absorbances of the free drug and that measured at each DNA concentration, respectively; C_D^0 and C_B are the total and bound drug concentrations, respectively.

Under the assumption previously discussed, that the absorption is due only to the free form of the compounds ($f_B = 0$), the concentrations of free and bound drug are given by:

$$C_B = C_D^0 \cdot \frac{A - A_0}{A_0}$$

$$C_F = C_D^0 - C_B$$

The experimental data were also fitted either to the linear Scatchard plot,[7]

$$\frac{r}{C_F} = (n - r) \cdot K$$

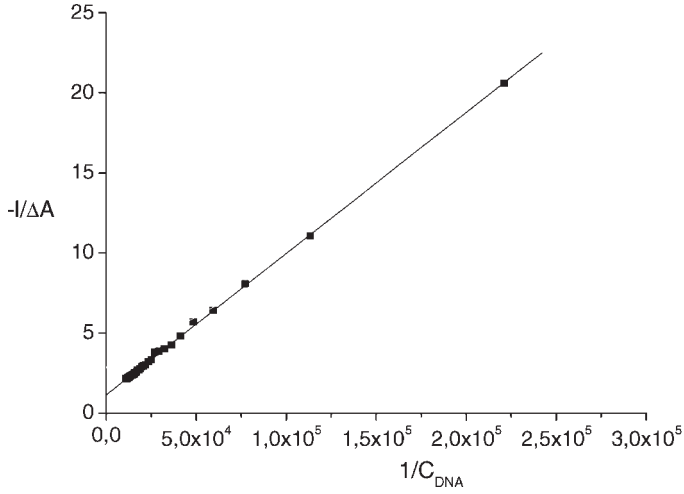

Figure 5.
Benesi-Hildebrand plot for norfloxacin - DNA system.

or to a non-linear regression:

$$r = \frac{n \cdot K \cdot C_F}{1 + K \cdot C_F}$$

corresponding to a single class of non-interacting binding sites that do not exhibit cooperative behaviour. In these relationships, r is the binding ratio ($r = \frac{C_B}{C_{DNA}}$), C_F is the free drug concentration, C_B is the bound drug concentration and n is the number of binding sites.

The Scatchard plots presented in Figure 6, attest the presence of two processes. The solid lines represent the best fit of the linear portion of the plot. This linear part with a negative slope, corresponding to polymer to drug ($\frac{P}{D}$) ratios in the range 0,5–3,5 is characteristic for non-cooperative binding to one class with n equivalent sites. Considering this linear segment, the binding constant $K = 0{,}94 \cdot 10^4 M^{-1}$ and the number of binding sites $n = \sim 1$ were obtained.

The form of the binding curves at the absorption maximum of the monomer ($\varepsilon_{app} = f(\frac{P}{D})$, at 275 nm) presents the features of a cooperative binding caused by stacking interaction of neighbouring bound drug molecules and may be interpreted in the frame of a theory developed by G. Schwarz.[10,14,15] This theory defines the fraction of free drug by:

$$\gamma_D^* = \frac{C_F}{C_D^0} = \frac{\varepsilon_{app} - \varepsilon_{st}}{\varepsilon_M - \varepsilon_{st}}$$

Plotting the apparent absorption coefficient $\varepsilon_{app,}$ at 275 nm, versus the reciprocal value of the total weighing-in concentration of the drug ($\frac{1}{C_D^0}$), at different constant polymer to drug ($\frac{P}{D}$) ratios lead to straight lines which converge to ε_{st} (Figure 7).

Extrapolation to $\frac{1}{C_D^0} \to 0$ yields the molar absorption coefficient of bound and stacked drug molecules ε_{st} as being the intercept on the ordinate axis, value practically the same for all $\frac{P}{D}$ ratios within our experimental errors $28000(\pm 250) M^{-1}cm^{-1}$.

We may determine the fraction of free drug γ_D^* as a function of polymer to drug ratio ($\frac{P}{D}$), at constant concentration of norfloxacin. This is carried out by evaluating the decrease of ε_{app} due to increasing the concentration of the polymer. An example is given in Figure 8.

Extrapolation of the limiting straight line to the axis of abscissas yields the reciprocal value of n, which stands for the number of binding sites per DNA segment (this yields $n = 0{,}67$ in our case). The second auxiliary straight line in the diagram has half the slope of the first one and corresponds

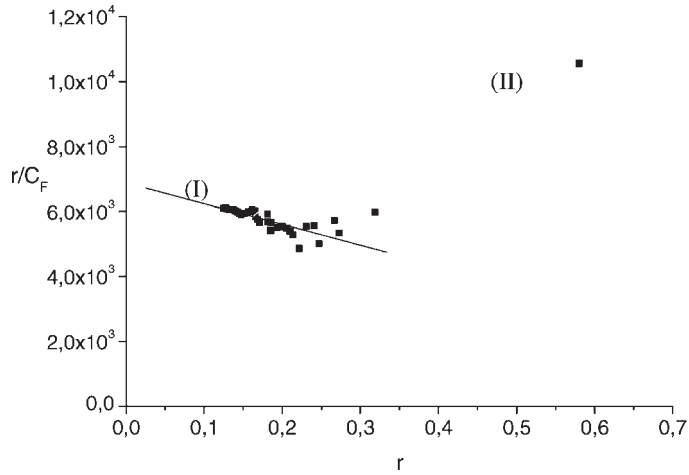

Figure 6.
Scatchard plot for norfloxacin - DNA system.

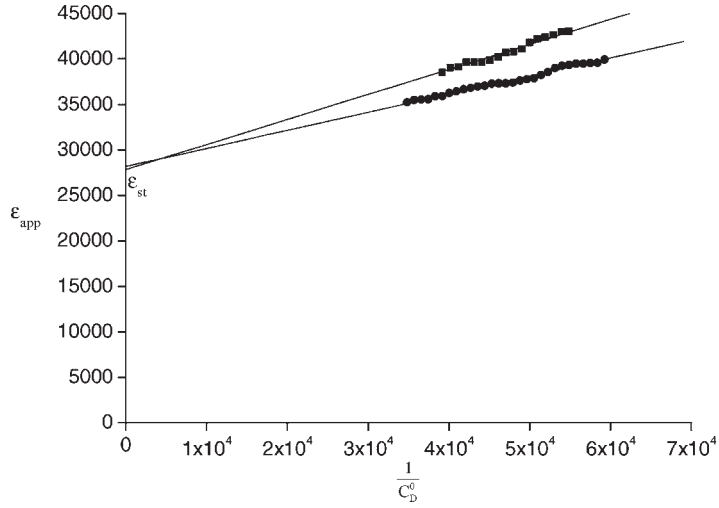

Figure 7.
The apparent absorption coefficient as a function of reciprocal value of the total weighing-in concentration of the norfloxacin.

to $\theta = \frac{1}{2}$, i.e. to the limiting case where no cooperativity is observed (the binding sites being alternatively occupied and not occupied).

The value of γ_D^* at the intersection point with the experimental curve, γ_D^o, may be used to calculate the binding constant K according to the equation[10]:

$$K = (\gamma_D^o \cdot c_D^o)^{-1} + 2K_d$$

valid if binding to the polymer is stronger than the dimerization tendency of the drug, $K \gg K_d$. In our case, we find $K \gg K_d$ so this is a good approximation.

Another useful experimental approach to evaluate binding data at large polymer to drug ratios was developed by Schwarz and Watanabe.[16] In any experiment, binding is usually measured by means of optical absorbance that changes when the free drug is bound to the polymer. One may

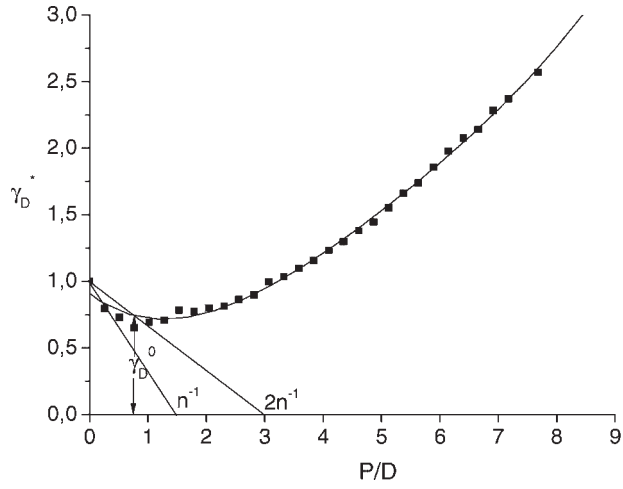

Figure 8.
The plot of γ_D^* versus $\frac{P}{D}$ ratio for norfloxacin - DNA system.

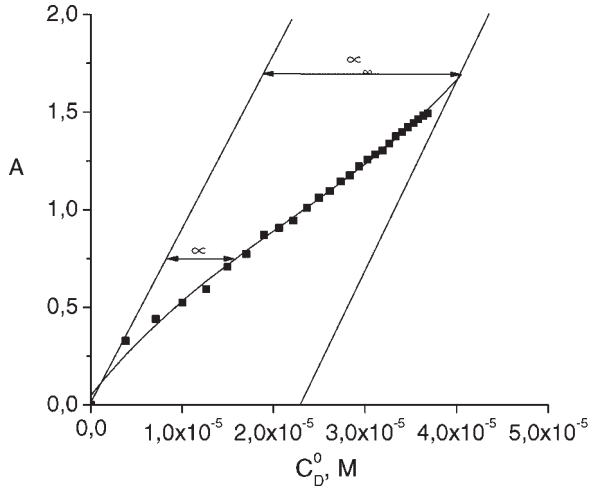

Figure 9.
Plot of absorbance versus total norfloxacin concentration.

ordinarily assume that absorbance depends linearly on the concentrations of free and bound drug, respectively. Thus, we write:

$$A = (\varepsilon_A \cdot C_A + \varepsilon_a \cdot C_a)l$$

with the molar absorption coefficient ε_A, ε_a referring to the two states of the drug.

The quantity ε_A can conveniently be obtained from the concentration dependence of A in a polymer free solution of the drug. An analogous determination of ε_a requires the availability of solution where all drug molecules are bound. In practice it may be difficult to realize this condition. A method of measurement and data processing starts from a drug free solution of polymer to which the drug is added bit by bit. In other words, the absorbance will be measured at constant polymer concentration and gradually increasing total drug concentration. A corresponding plot of absorbance versus total norfloxacin concentration is presented in Figure 9. If the polymer concentration is equal to zero then the absorbance follows the zero-line. The deviations from this zero-line indicate binding of drug. When norfloxacin concentration has finally been increased so much that all available polymer is saturated the curve runs parallel to zero-line. There we have a constant $\propto$, denoted $\propto_\infty$. Thus the degree of binding θ is equal to:

$$\theta = \frac{\alpha}{\alpha_\infty}$$

where $\propto$ and $\propto_\infty$ are defined in the Figure 9.

We applied the method of Schwarz and Watanabe[16] to determine the cooperative binding constant (K) and the cooperative interaction parameter (q), from absorption measurements by using the equation:

$$\frac{2\theta - 1}{\sqrt{\theta(1-\vartheta)}} = \sqrt{\frac{q}{n}}(K \cdot C_D^0 - 1)$$

From the linear plot of $\frac{2\theta-1}{\sqrt{\theta(1-\theta)}}$ against drug concentration (Figure 10), we obtained a cooperative binding constant equal to $4{,}52 \cdot 10^4 \mathrm{M}^{-1}$ and a value of 6–8 for the cooperative interaction parameter.

where l is the path length, ΔA is the change in the absorbance, $\Delta\varepsilon$ is the molar absorptivity difference ($\Delta\varepsilon = \varepsilon_B - \varepsilon_F$), ε_F and ε_B are the free and bound drug absorption coefficients, C_D^0 is the total concentration of drug, C_F and C_B are the free and bound drug concentrations, C_{DNA} is the concentration of DNA, K is the binding constant, r is the binding ratio, n is the number of binding sites.

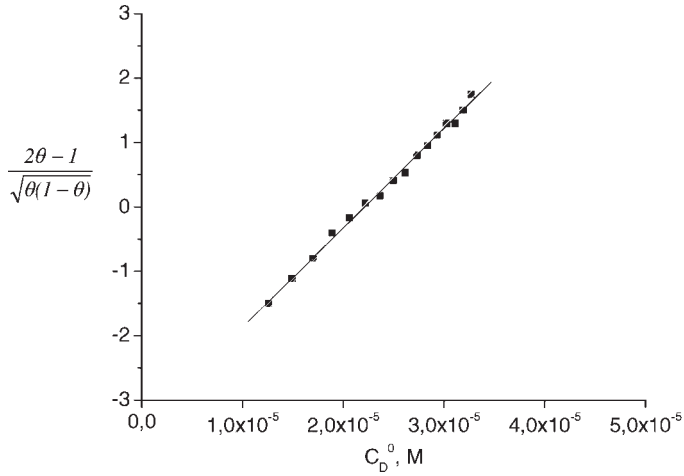

Figure 10.
Plot of $\frac{2\theta-1}{\sqrt{\theta(1-\theta)}}$ versus total norfloxacin concentration.

Conclusion

The binding of norfloxacin to calf thymus DNA produces hypochromism, broadening of the envelope and a red-shift in the drug absorption region, the characteristics that indicated that norfloxacin interacts with DNA. Based on the isosbestic point observed in norfloxacin - DNA system and the assumption that at a relatively low concentration of norfloxacin and DNA the formation of higher order complexes is unlikely, it can be concluded that norfloxacin forms a 1:1 homogeneous ground state complex with DNA.

The analysis of the norfloxacin interaction with calf thymus DNA, using several methods points out two binding types for the norfloxacin - DNA system: a non-electrostatic (internal) type consisting of the intercalation of the drug between the base-pairs from DNA and an external type, cooperative where the electrostatic interactions with the phosphate groups of DNA are predominant. The first binding process analysed by Benesi-Hildebrand, Scott and Scatchard models suppose a 1:1 binding ratio and do not account explicitly for either the dimerization of the drug or cooperativity effects on the binding. The second binding process analysed by Schwarz and Watanabe-Schwarz methods, supposes a linear lattice of equivalent binding sites with nearest neighbour cooperativity.

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