Absorption study of ofloxacin - DNA system

L. E. VÎJAN*
University of Piteşti, Faculty of Sciences, Târgu din Vale No.1, Piteşti - 110040, Romania

Fluoroquinolone carboxylic acids are antibacterial agents that attack DNA gyrase and topoisomerase IV on chromosomal DNA. In this work, the absorption spectra of ofloxacin in the presence of different amounts of calf thymus DNA are presented and discussed. The self-association of ofloxacin in aqueous solutions was considered in terms of the dimerization equilibrium and the dimerization parameters were determined. The results of the interaction between ofloxacin and DNA were rationalized in terms of the Benesi-Hildebrand, Scott, Scatchard, Wolfe and Watanabe-Schwarz models, in order to obtain the binding parameters.

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1. Introduction

Fluoroquinolone carboxylic acids are a class of chemotherapeutic agents with antibacterial activity used in human and veterinary medicines for treat urinary and respiratory tract infections. These antibiotics inhibit the action of type II topoisomerase that catalyzes the conversion of relaxed supercoiled DNA into a negatively supercoiled form [1,2]. In addition, many bacteria are sensitive to the fluoroquinolones: some pathogenic species, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*, readily acquire resistance mutations that severely limit fluoroquinolone usefulness [3].

Ofloxacin (9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperanzinyl)-7-oxo-7H-pyrido[1,2,3-δε]-1,4-benzoxazine-6 carboxylic acid) is a member of the fluoroquinolone family. It possesses a tricyclic structure and has a methyl group at the C-3 position in the oxazine ring, resulting in an asymmetric centre at this position. It is a zwitterionic compound with acid dissociation constants of 6.08 (pK_{a1}) and 8.25 (pK_{a2}) [4,5] (Fig. 1). As shown that this antibiotic is primarily cationic below pK_{a1} (N-4 in the piperazinyl group), anionic above pK_{a2} (3-carboxyl group) and zwitterionic (net neutral) between pK_{a1} and pK_{a2} [6].

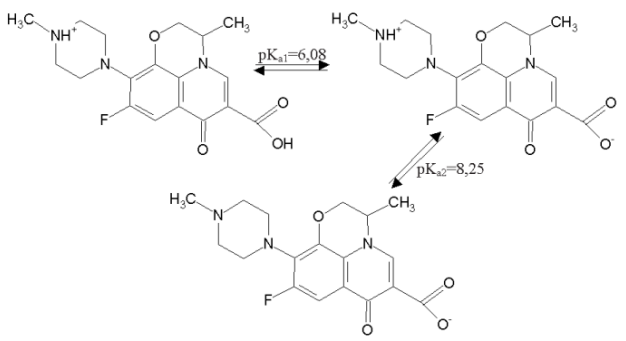

Fig. 1. The ionization of ofloxacin.

It was reported that ofloxacin, one of the DNA gyrase inhibitors of the fluoroquinolone family, does not directly bind to gyrase but forms the stabile complexes with DNA or polynucleotides [7,8]. Lee and coworkers have found that S-ofloxacin forms a complex with DNA much more efficiently than *R*-enantiomer [7]. From the changes in fluorescence intensity at 453.5 nm, through the agency of the Benesi-Hildebrand plots, they have found the following values for the association constant for ofloxacin - DNA complex: $1.4 \cdot 10^3$ M^{-1} for *R*-enantiomer and $8.2 \cdot 10^3$ M^{-1} for *S*-enantiomer [7,9]. More recently, the circular dichroism and fluorescence studies were performed on *S*- and *R*-ofloxacin complexed with various synthetic polynucleotides [8]. From these studies, the following conclusions were reported:

(1) *S*-ofloxacin forms a complex with poly [d(A-T)$_2$] and poly[d(I-C)$_2$] while *R*-enantiomer does not,

(2) poly[d(G-C)$_2$] can form a complex with both enantiomers of ofloxacin, but the complex formation with *S*-ofloxacin is far more effective,

(3) in the complex, both enantiomers of ofloxacin conceivably sit in the minor groove with possibility of partial intercalation.

In the present work, the absorption spectra of ofloxacin (the racemic form of drug), in the presence of different amounts of calf thymus DNA are presented and discussed. This study points to following issues: the self-association of drug, in order to determine the molar absorption coefficient of the monomer (ε_M), the molar absorption coefficient of the dimer (ε_D), the dimerization constant (K_d), and the interaction of drug with DNA, in order to determine the number of binding sites per DNA segment (n) and the binding constant (K).

The results of self-association of ofloxacin were rationalized in terms of two methods, Tipping and Schwarz [10,11]. The results of ofloxacin – calf thymus DNA system were rationalized in terms of five methods,

Benesi-Hildebrand, Scott, Scatchard, Wolfe and Watanabe-Schwarz [9,12-15].

2. Experimental

Ofloxacin (the racemic form of drug) and calf thymus DNA were purchased from Sigma-Aldrich Company (St. Louis, USA).

The stock solutions of ofloxacin and DNA were prepared by dissolving commercially purchased reagents in doubly distilled water. The concentrations of the stock solutions were determined by the molar absorption coefficients: ε_{288nm}= 30500 $M^{-1}cm^{-1}$ for ofloxacin [7] and ε_{260nm}= 6600 $M^{-1}cm^{-1}$ for DNA [16].

All experiments were carried at pH neutral. The values of the absorbance were read at 288 nm, the wavelength that corresponds to maximum of absorption of the monomer form of the drug.

The analysis of the experimental data obtained from the absorption spectra was made using the OriginPro7 program.

The absorption measurements were performed on a Perkin-Elmer Lambda 25 UV-VIS spectrophotometer using the 1 cm optical path length quartz cell, at room temperature.

3. Results and discussion

The UV-Vis spectra of ofloxacin show a major band centred on 288 nm and three minor bands centred around 230, 250 and 335 nm. It may be noted that the relative intensities of the bands vary upon dilution. The ratio of absorbance A_{230}/A_{288}, A_{250}/A_{288} and A_{335}/A_{288} has been applied as a measure of the degree of drug self-association.

The literature describes several computation methods for estimating the self-association constant [10,11]. Thus, starting from the monomer - dimer equilibrium:

$$2M \rightleftharpoons^{K_d} D$$

and the following equations:

$$K_d = \frac{[D]}{[M]^2} \quad (1)$$

$$A = \varepsilon_{app} \cdot C^0 \cdot l = (\varepsilon_M \cdot [M] + 2\varepsilon_D \cdot [D]) \cdot l \quad (2)$$

where ε_{app}, ε_M and ε_D denote the molar absorption coefficients of the solution (at 288 nm), the pure monomer and the pure dimer, respectively, C^0 is the total concentration of drug, [M] and [D] are the concentrations of the monomer and dimer, respectively. The mass conservation requires:

$$C^0 = [M] + 2[D] \quad (3)$$

Tipping [10] and Schwarz [11] have developed a theory for estimating the self-association parameters. Elimination of [M] using (1) and (3), respectively (2) and (3), followed by elimination of [D] yields the Tipping expression:

$$\sqrt{\frac{C^0}{\varepsilon_M - \varepsilon_{app}}} = \frac{1}{\varepsilon_M - \varepsilon_D}\sqrt{C^0 \cdot (\varepsilon_M - \varepsilon_{app})} + \sqrt{\frac{1}{2K_d \cdot (\varepsilon_M - \varepsilon_D)}} \quad (4)$$

So, through the agency of a linear plot of $\sqrt{\frac{C^0}{\varepsilon_M - \varepsilon_{app}}}$ against $\sqrt{C^0 \cdot (\varepsilon_M - \varepsilon_{app})}$, we have calculated K_d, ε_M and ε_D.

In addition, by combining the equations (1), (2) and (3), [M] and [D] can be eliminated and the result may be represented as:

$$\sqrt{\frac{\varepsilon_M - \varepsilon_{app}}{c^o}} = \sqrt{\frac{2K_d}{\Delta\varepsilon}} \cdot [\Delta\varepsilon - (\varepsilon_M - \varepsilon_{app})] \quad (5)$$

where $\Delta\varepsilon$ is the difference in the molar absorption coefficients of the monomer and dimer. So, by the agency of a Schwarz plot [11] of $\sqrt{\frac{\varepsilon_M - \varepsilon_{app}}{c^o}}$ versus (ε_M-ε_{app}), from the intercept and slope, $\Delta\varepsilon$ and K_d have been calculated. Applying the two methods described in literature, Tipping and Schwarz [10,11], we have obtained a value of 2420(±180) M^{-1} for the dimerization constant and a value of 15500(±580) $M^{-1}cm^{-1}$ for the molar absorption coefficients of dimer, a value of 30500 $M^{-1}cm^{-1}$ being imposed for the molar absorption coefficients of monomer.

A family of curves obtained at the titration of ofloxacin solutions of concentrations in the range 10^{-6}-10^{-5}M with calf thymus DNA is presented in fig. 2. It may be noted that the relative intensities of the major bands vary upon the polymer to drug ratios (P/D). The isosbestic points observed in this system, implying the homogeneous conformation of the ofloxacin molecule (in our case, the zwitterionic ofloxacin) bound to calf thymus DNA, *i.e.*, the system consists only the DNA free and DNA bound ofloxacin.

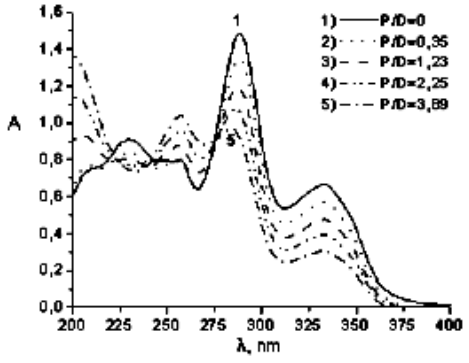

Fig. 2. Absorption spectra of ofloxacin - DNA system.

The binding constant for ofloxacin - DNA system can be represented by the following equilibrium:

$$\text{ofloxacin} + \text{DNA} \overset{K}{\rightleftharpoons} \text{complex}$$

through the agency of the changes in absorbance at a fixed wavelength (288 nm), using different methods. Considering this equilibrium, the absorbance is assumed to be the sum of the absorbance of the free and bound species, weighted by their respective concentrations:

$$A = f_0 \cdot (C^0 - C_B) + f_B \cdot C_B \quad (6)$$

$$A_0 = f_F \cdot C^0 \quad (7)$$

where A_0 and A are the absorbencies of the free drug and that measured at each DNA concentration, C^0, C_B and C_F are the free, bound and total drug concentrations.

Under the assumption previously discussed in this paper, 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:

$$C_B = C^0 \cdot \frac{A - A_0}{A_0} \quad (8)$$

$$C_F = C^0 - C_B \quad (9)$$

We have evaluated the binding constant from different methods, proposed by Benesi-Hildebrand, Scott, Scatchard and Wolfe [9,12-14]. The experimental data lead to the linear Benesi-Hildebrand, respectively Scott, Scatchard and Wolfe plots, an example being presented in fig. 3 for ofloxacin – DNA system.

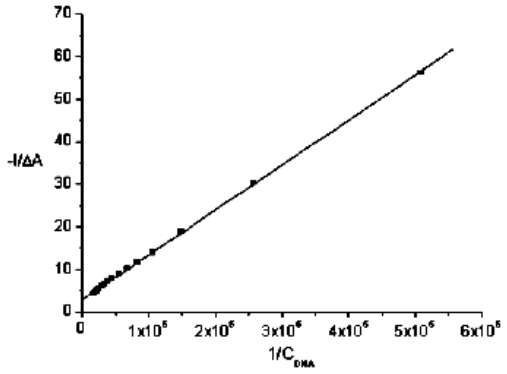

Fig. 3. Benesi-Hildebrand plot of ofloxacin binding to DNA.

The equations utilized and the results obtained for the interaction of ofloxacin with calf thymus DNA are summarized in Table 1.

Table 1. Binding parameters for ofloxacin - DNA system.

Method	Equations	R	$K \cdot 10^{-4}$, M^{-1}	n
Benesi-Hildebrand	$\frac{1}{\Delta A} = \frac{1}{C^0 \cdot K \cdot \Delta\varepsilon} \cdot \frac{1}{C_{DNA}} + \frac{1}{C^0 \cdot \Delta\varepsilon}$	0.9998	2.75	-
Scott	$\frac{l \cdot C_{DNA}}{\Delta A} = \frac{1}{C^0 \cdot \Delta\varepsilon} \cdot C_{DNA} + \frac{1}{C^0 \cdot K \cdot \Delta\varepsilon}$	0.9923	2.25	-
Scatchard	$\frac{\Delta A}{l \cdot C_{DNA}} = -\frac{K}{l} \cdot \Delta A + C^0 \cdot K \cdot \Delta\varepsilon$	0.9842	2.83	-
	$\frac{r}{C_F} = (n - r) \cdot K$	0.9924	2.69	0.36
	$r = \frac{n \cdot K \cdot C_F}{1 + K \cdot C_F}$	-	2.61	0.45
Wolfe	$\frac{C_{DNA}}{\Delta\varepsilon_{app}} = \frac{C_{DNA}}{\Delta\varepsilon} + \frac{1}{K \cdot \Delta\varepsilon}$	0.9961	27.7	-
Watanabe-Schwarz	$\frac{2\theta - 1}{\sqrt{\theta(1-\theta)}} = \sqrt{\frac{q}{n}}(K \cdot C_D^0 - 1)$	0.9963	10.6	-

where: ε_{app}, ε_F, ε_B are the apparent, free and bound drug absorption coefficients, l is path length, ΔA - the change in the absorbance at a given wavelength, C^0 - the total concentration of drug, C_{DNA} - the concentration of calf thymus and $\Delta\varepsilon$ - the molar absorptivity difference.

The processing of the ofloxacin – calf thymus DNA data by Benesi-Hildebrand, Scott and Scatchard methods show close values of the binding constant, these values being with a less order of measures than the value obtained by Wolfe method.

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

$$\frac{r}{C_F} = (n - r) \cdot K \tag{10}$$

or to a non-linear regression:

$$r = \frac{n \cdot K \cdot C_F}{1 + K \cdot C_F} \tag{11}$$

corresponding to a single class of non-interacting binding sites that do not exhibit cooperative behaviour. In these relationships, *n* is the number of binding sites and *r* is the binding ratio:

$$r = \frac{C_B}{C_{DNA}} \tag{12}$$

The Scatchard plots, presented in Fig. 4, attest the presence of two binding processes: the process (I), corresponding at small values of P/D ratios, respectively the process (II), corresponding at medium values of P/D ratios. From the solid line corresponding to process (II), characterized by a negative slope, the binding constant $K=2.69(\pm0.22)\cdot10^4$ M^{-1} and the number of binding sites n=~0.36(±0.02) were found.

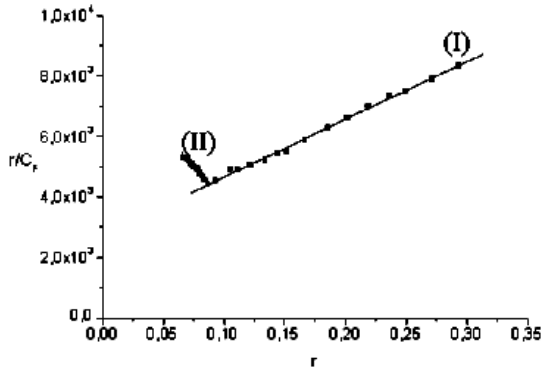

Fig. 4. Scatchard plot of ofloxacin binding to DNA.

The non-linear fitting of both processes (equation 11, Fig. 5) yield similar binding parameters for the process (II): $K=2.61(\pm0.16)\cdot10^4$ M^{-1} and n=0.45(±0.03). These values are similar with those obtained in Benesi-Hildebrand, Scott and Scatchard methods.

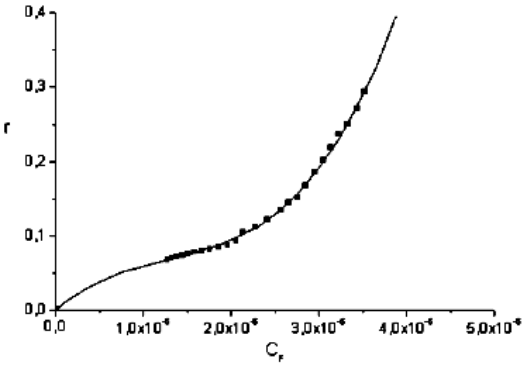

Fig. 5. Non-linear fitting of both binding processes.

Another useful experimental approach to evaluate binding data at large polymer to drug ratios was developed by Schwarz and Watanabe [15]. This theory assumes two processes:

(1) formation of a binding complex with no nearest binding sites occupied (nucleation), characterized by a binding constant $K^{\#}$;

(2) formation of a binding complex at a site where the neighbour site is already occupied (aggregation), described by a binding constant *K*.

The ratio:

$$q = \frac{K}{K^{\#}} \tag{13}$$

measures the degree of cooperativity.

In any experiment, the binding is usually measured by means of optical absorbance that changes when the free drug is bound to the polymer. One may ordinarily assume that absorbance depends linearly on the concentrations of free and bound drug, respectively. Thus, we write:

$$A = (\varepsilon_A \cdot C_A + \varepsilon_a \cdot C_a) \cdot l \tag{14}$$

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 plot of absorbance versus total ofloxacin concentration is presented in Fig. 6. 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 ofloxacin 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:

$$\theta = \frac{\alpha}{\alpha_\infty} \tag{15}$$

where $\propto$ and $\propto_\infty$ are defined in Fig. 6.

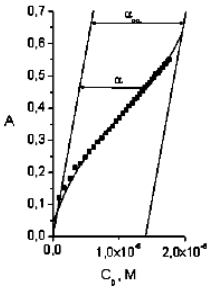

Fig. 6. Plot of absorbance versus total ofloxacin concentration.

From the linear plot of $\frac{2\theta-1}{\sqrt{\theta(1-\theta)}}$ versus total ofloxacin concentration (Fig. 7), the cooperative binding constant, the equilibrium constant of the nucleation process and the cooperative interaction parameter were obtained. In terms of the Schwarz and Watanabe theory, the binding constant has the same order of measures than in Wolfe theory.

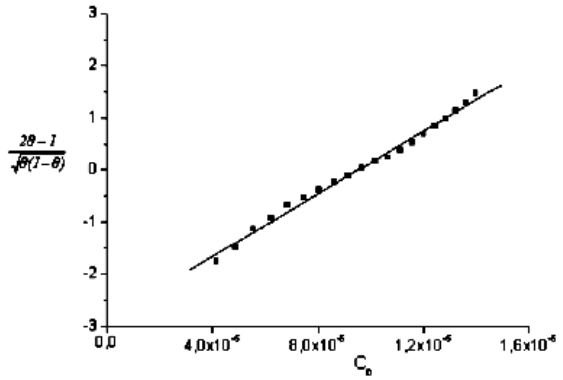

Fig. 7. Watanabe-Schwarz plot of ofloxacin binding to DNA.

4. Conclusions

Based on the isosbestic points observed in ofloxacin – calf thymus DNA system and assuming that at a relatively low concentration of ofloxacin and DNA, the formation of higher order complexes is unlikely, it can be concluded that ofloxacin forms a 1:1 homogeneous ground state complex with DNA.

The spectral binding data of ofloxacin to DNA point out two distinct types of interactions: 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, supposes a 1:1 binding ratio and does not account explicitly for either the dimerization of the drug or cooperativity effects on the binding. The second binding process analysed by Wolfe and Watanabe-Schwarz methods, supposes a linear lattice of equivalent binding sites with nearest neighbour cooperativity.

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*Corresponding author: loredana.vijan@upit.ro
vloredana2005@yahoo.com