

pH에 따른 Norfloxacin의 형태 및 DNA와의 상호작용에 관한 연구

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Protonation State of Norfloxacin and Their Interaction with DNA

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요 약. 여러 가지의 분광법과 전위차 적정법을 이용하여, 퀴놀론의 유도체인 norfloxacin의 자체 회합과 pH에 따른 형태에 대하여 연구하였다. norfloxacin의 작용기 중에서 피페라진 고리와 카르복실기의 두 질소 원자는 낮은 pH 용액에서는 수소화(양이온 형태)되고, 높은 pH 용액에서는 두 수소가 모두 이탈하며(음이온 형태), 중간 pH 범위의 용액에서는 zwitter 이온이 두드러지게 형성되었다. 또한, 이 중간 pH용액에서는 norfloxacin 두 분자가 자체 회합을 이루었다. Stern-Volmer 측정법에 의하여 norfloxacin-DNA 결합체의 결합 상수를 조사하였는데, 용액의 pH가 낮을수록 그 결합 상수는 증가하였다. 이것은 용액 상태에서 DNA에 결합하는 norfloxacin의 분자종이 여러 분자종 중에서 그 형태가 양이온임을 나타내는 것이다.

ABSTRACT. We investigated the self-association and protonation state of norfloxacin, a member of quinolone antibiotics, using electric absorption and potentiometric titration. Both nitrogen at the piperazine ring and carboxylic acid were protonated at a low pH (cationic norfloxacin), and deprotonated at a high pH (anionic norfloxacin). In the intermediate pH range, a neutral species was dominant with the possibility of forming a zwitter ion. We also observed that norfloxacin molecules can be stacked to form a dimer at an intermediate pH. The equilibrium constant of the norfloxacin-DNA complex formation, which was measured by Stern-Volmer method, increases as the pH of the system is lowered. This observation indicates that it is the cationic norfloxacin that forms a complex with DNA among various norfloxacin species in aqueous solution.

INTRODUCTION

Quinolones are a group of well known antibiotics. A large amount of biological data indicates that the functional target of these drugs is DNA gyrase, which catalyzes the conversion of relaxed supercoiled DNA into a negatively supercoiled form. However, it was found that norfloxacin, one of the most potent DNA gyrase inhibitors of the quinolone family, does not directly bind to

DNA gyrase but binds to DNA itself.¹ Subsequent studies by the same group²⁻⁴ proposed a cooperative quinolone-DNA binding model in the inhibition site of DNA gyrase in the presence of ATP. In this model, the norfloxacin molecules were bound in the specific single-stranded DNA pocket that was induced by gyrase and were stabilized by π - π stacking of the norfloxacin rings and tail to tail hydrophobic interactions. In contrast, in the presence of an appropriate amount Mg^{2+} ions, norfloxacin binds to plasmid DNA in such a manner that the

Mg²⁺ ion acts as a bridge between the phosphate groups of the nucleic acids and the carbonyl and carboxyl moiety of norfloxacin.⁵ Our study on the base (sequence) specificity and binding geometry of the DNA-norfloxacin complex using fluorescence and linear dichroism spectroscopy demonstrated that norfloxacin binds preferentially to G-C base pairs, and that the molecular plane of the norfloxacin is nearly perpendicular to the DNA helix axis.^{6,8} It has also been recently demonstrated that quinobenzoxazine, a quinolone family, forms a binary complex with Mg²⁺ and a tertiary complex with Mg²⁺ and DNA.^{9,11}

The behavior of norfloxacin in an aqueous solution, *viz.*, the protonation state and self stacking properties, was reported by few investigators.¹²⁻¹⁴ It is possible for norfloxacin to have various protonated states, since there are at least two possible protonation sites. The *pK_a* values for the carboxylic acid and the nitrogen atom at piperazine ring were reported to be 6.3 and 8.5, respectively.¹² Therefore, at least four differently protonated norfloxacin species can exist at pH 7.0. Norfloxacin (and quinolones in general) is a polycyclic aromatic hydrocarbon that consists of a drug-enzyme interaction domain, a DNA-hydrogen bonding domain, and a drug-drug self-assembly domain. Polycyclic aromatic hydrocarbons have a tendency to associate by themselves in an aqueous solution. In addition, norfloxacin contains functional moieties—namely, carbonyl and a carboxylic acid group in the DNA-hydrogen bonding domain—to which a proton-mediated self-association is possible. Therefore, norfloxacin can form a higher order self-associated form in an aqueous solution by stacking between the polycyclic aromatic part and the association between the carboxylic acid in addition to various protonated states.

The questions addressed in this work include how norfloxacin is protonated and whether (and how) it is self-associated in an aqueous solution. In addition, we examined how, if norfloxacin is self-associated, do the dimer and higher associated forms of norfloxacin affect the binding of norfloxacin to DNA, and which protonated species forms a complex with DNA.

EXPERIMENTAL

Materials. Calf thymus DNA (referred to as DNA)

was purchased from Sigma and dissolved in a 5 mM cacodylate buffer at pH 7.0 containing 100 mM NaCl and 1 mM EDTA. The solution was then centrifuged at 10,000 rpm for one hour to remove any insoluble solids that may have been present. NaCl and EDTA were removed by dialyzing against 5 mM cacodylate buffer at pH 7.0, utilizing a Sigma dialyzing bag with a 10,000 molecular weight cutoff. The buffer was changed every five hours for a total of five times. All processes were performed at 4°C. The norfloxacin was purchased from Sigma and used without further purification. The concentrations of DNA and norfloxacin were determined spectrophotometrically using the extinction coefficients $\epsilon_{270nm}=37,500 \text{ cm}^{-1}\text{M}^{-1}$ for norfloxacin and $\epsilon_{250nm}=6,700 \text{ cm}^{-1}\text{M}^{-1}$ for DNA. All absorption spectra were recorded either on a Jasco V-550 or a Hewlett Packard 8452A diode array spectrophotometer. The cells with 0.1, 1.0, 10.0 cm path lengths were used for absorption measurements as necessary.

Measuring the equilibrium constant using the Stern-Volmer method. The equilibrium constant of norfloxacin with DNA was measured using the well-known Stern-Volmer method:¹⁵

$$F_0/F = 1 + K_{SV} [DNA]$$

where F_0 and F denote the fluorescence intensity of the given fluorophore in the absence and presence of a quencher. Two mechanisms, the dynamic and static processes, may explain the simple fluorescence quenching process.¹⁵ In the dynamic quenching mechanism, the energy of the excited fluorophore transfers to the quencher when they collide. As the temperature of the system increases, the quenching rate is increased because the number of collisions increases, and the absorption spectrum of the fluorophore is not changed because the quencher interacts only with the excited fluorophore. In the static quenching mechanism, the fluorescence is quenched by forming a ground state non-fluorescent fluorophore-quencher complex, hence a decreasing temperature results in an enhancement in the quenching efficiency, because the complex is usually stable at low temperatures. The absorption spectrum of the fluorophore is changed upon adding the quencher in the static quenching mechanism. The fluorescence spectra were obtained by a Perkin Elmer LS 50B fluorometer. For all

fluorescence quenching measurement, the samples were excited at 323 nm and the intensities were measured at 415 nm. Slit widths were 4 and 7 nm for excitation and emission window.

Potentiometric titration. The potentiometric titration was performed for norfloxacin (0.068 m mol/100 mL) at 30 °C following the method of Martell *et al.*¹⁶ A 3.28×10^{-2} M KOH and 3.28×10^{-2} M HCl solution was used as the standard solution.

RESULT

pH dependence of the absorption spectra of norfloxacin and pH titration. pH dependence of the absorption spectra of norfloxacin and pH titration. The absorption spectra of DNA-free norfloxacin at various pH are depicted in Fig. 1. Our result is in agreement with already reported changes in absorption spectrum of norfloxacin upon the pH change.¹²⁻¹⁴ The absorption band at 272 nm at pH 7.0 gradually red-shifts and exhibits hyperchromism as the pH of the solution decreases. At pH 4.0, this band has shifted to 276 nm. In contrast, both bands above 300 nm exhibit a hypochromism, blue-shift, and broadening with a decreasing pH. Three isosbestic points, at 272 nm, 318 nm and 344 nm, were found, indicating that two major light-absorbing norfloxacin species were present in the solution between pH 4.0 to 7.0. As the pH increases to 8.0, the absorption pattern is similar to that at pH 7.0. These observations ensure that the protonated state of the norfloxacin can affect the absorption pattern.

Two pK_a values, 6.3 and 8.5, were found for norflox-

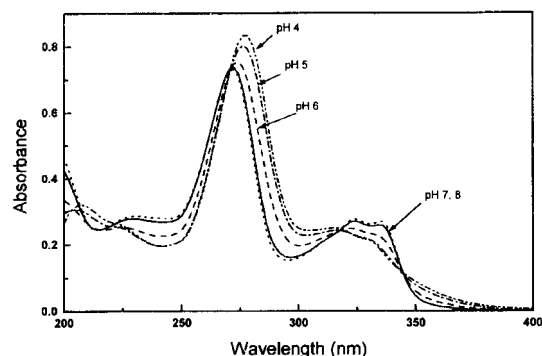
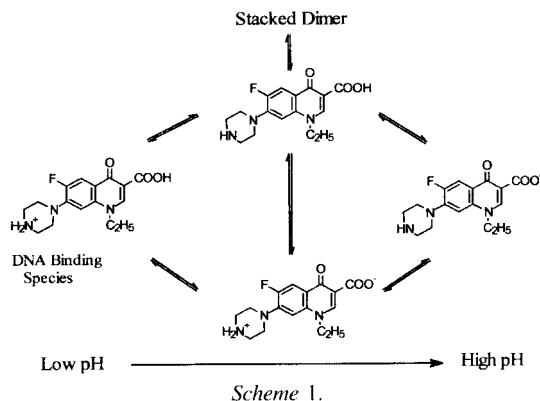


Fig. 1. Absorption spectrum of norfloxacin (20 mM) at various pH.



acin from the acid-base titration experiment (data not shown) and these values are in agreement with reported ones.¹² At a pH value higher than 10.5, the carboxylic acid moiety of all norfloxacin is probably deprotonated and the nitrogen atom at the end of the piperazine ring mono-protonated (Scheme 1). The charge of the whole molecule is -1 in this pH range. This species may not bind to DNA because only electrically positively charged or neutral drugs preferentially bind to DNA. In fact, the binding of norfloxacin to DNA was negligible at a high pH (see below). Consequently, this species will not be discussed further. At the pH range lower than 4.5, the carboxylic acid is mono-protonated and the nitrogen atoms at the end of the piperazine ring are di-protonated, resulting in a total charge of +1. Four species may co-exist in the intermediate pH range, including the species in which both protonation sites are mono-protonated (neutral species) and the zwitter ionic form of this species. The zwitter ion form is dominant at pH 7.0.¹²

Dependence of norfloxacin absorption spectrum on the polarity of the solution and the concentration. The concentration-dependent norfloxacin absorption spectrum is depicted in Fig. 2. The spectra are shown for concentrations of 3.06 μ M, 30.6 μ M, and 306 μ M. The absorption spectrum of the highest concentration was collected using a 1 mm cell and multiplied by ten. All the spectra were then normalized to the highest concentration for easy comparison. Interestingly, we found that the concentration-dependent absorption changes are similar to the pH-dependent absorption changes (Figs. 1 and 2). The absorption spectrum measured at the low con-

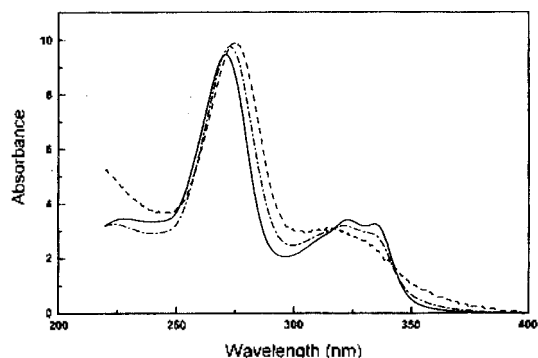


Fig. 2. Absorption spectrum of norfloxacin at various concentrations in aqueous solution (3.06 μM ; dashed curve, 30.6 μM ; dashed-dotted curve, and 306 μM ; solid curve). The absorption spectrum at the highest concentration was recorded using a 1 mm path length and multiplied by ten. All spectra were then normalized to the highest concentration.

centration was similar to that obtained at a low pH, and that at the high concentration resembled the high pH, suggesting that the absorption pattern at the low concentration represent the monomeric norfloxacin and that at the high concentration denotes the dimer. The formation of higher order complexes can be disregarded based on the observation of two isosbestic points at 316 nm and 341 nm; hence, only the monomer vs. dimer will be discussed.

Two forms of the dimer are possible in the norfloxacin case—a stacked dimer or a dimer connected *via* the electrostatic interaction of the carboxylic acids (which may be bridged by hydrogen). In the former, the dimers are stacked vertically and stabilized by the interaction between the π electrons in the aromatic rings. In the latter, norfloxacin can be associated by forming hydrogen bonds between the carboxylic acids of the neighboring molecules. If the norfloxacin molecules are stacked to form a dimer, it is stabilized as the environmental polarity increases. However, if the dimer is formed by electrostatic interaction, enhancement in the environmental polarity would result in destabilization of the dimer. The absorption patterns of norfloxacin in ethanol and water are compared in Fig. 3. The absorption spectrum in water resembles that at a high pH and high concentration. That recorded in ethanol is similar to the absorption pattern obtained from the low concentration, indicating that the dimers are dominant in a polar solution. Since

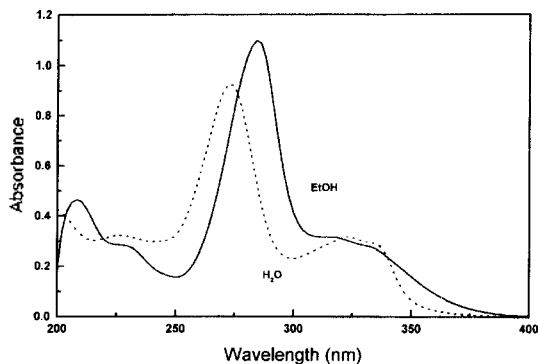


Fig. 3. The absorption spectrum of norfloxacin (25 μM) in water (dotted curve) and in ethanol (solid curve) in 5 mM cacodylate buffer at pH 7.0.

the dimer is more stable in a polar solution, norfloxacin may form the dimer in a stacked manner. The long wavelength region of the absorption spectrum of norfloxacin was remained to be unchanged upon adding urea (up to 5 M), supporting this conclusion (data not shown).

pH dependent equilibrium constants for norfloxacin-DNA complex formation. The fluorescence intensity of the norfloxacin decreased as the DNA concentration increased. An example is given in Fig. 4a. Furthermore, the fluorescence intensity in the presence of highly concentrated DNA was negligible, allowing us to measure the Stern-Volmer constant directly from decreases in the fluorescence intensity. We observed both changes in the

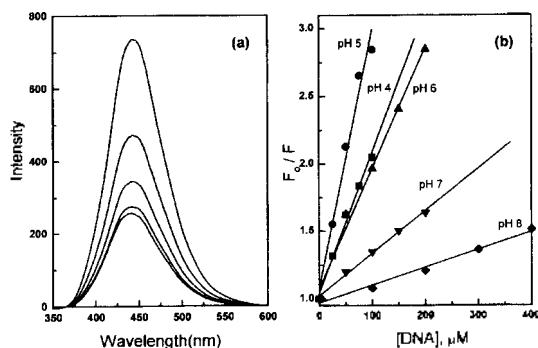


Fig. 4. (a) Change in fluorescence emission spectrum of norfloxacin with increasing DNA concentration. From top, DNA concentrations are 0, 25, 50, 75 and 100 μM . Norfloxacin was excited at 323 nm and the slit widths are 3 nm and 7 nm for excitation and emission windows. [norfloxacin]=1.0 μM . (b) pH dependent Stern-Volmer plot of norfloxacin in the presence of DNA.

Table 1. Stern-Volmer quenching constants for norfloxacin-DNA complex formation at various pH

pH	K_{sv} (M^{-1})
4.0	1.0×10^4
5.0	1.9×10^4
6.0	9.0×10^3
7.0	3.0×10^3
8.0	1.3×10^3

absorption spectrum of norfloxacin in the presence of DNA⁶ and a reduced quenching rate at higher temperatures (data not shown), ensuring that quenching occurs via the static mechanism. The Stern-Volmer quenching constant, K_{sv} , therefore, can be understood as the equilibrium constant for norfloxacin-DNA complexation. As discussed earlier, the fluorescence of the norfloxacin is quenched by forming a non-fluorescent ground state complex. Since several forms of norfloxacin, in terms of protonation state as well as stacking, can exist in an aqueous solution, identifying the norfloxacin species that bind most efficiently to DNA is of interest. Equilibrium constants for the norfloxacin-DNA complex were measured at various pH using the Stern-Volmer method (Fig. 4b and Table 1). The equilibrium constant at pH 8.0, at which the deprotonated (negatively charged) species and neutral species of norfloxacin are dominant, was quite low, at $1.3 \times 10^3 M^{-1}$, indicating that these species do not efficiently bind to DNA. The slope in the Stern-Volmer plot become stiffer as the pH of the system decreases from 8.0 to 5.0. The equilibrium constant at pH 5.0 is larger than that at pH 8.0 by a factor of about 14. The equilibrium constant reaches its highest value at pH 5.0, then, surprisingly, decreases as the pH is further lowered to 4.0, indicating that another mechanism besides the cationic monomer-zwitter ionic dimer scheme (and deprotonation-protonation scheme) contributes to the equilibrium state at a low pH.

DISCUSSION

Behavior of norfloxacin in an aqueous solution. A pH dependent norfloxacin structure appears in Scheme 1. As the pH increases from 4.0 to 7.0, the proton, which is located at the carboxylic group, is removed and the mono-cationic norfloxacin molecule becomes electrically neutral. At a neutral pH, transfer of the proton from

piperazine ring to the carboxylic acids to form a zwitter ionic isomer is possible. The shape of the absorption pattern at pH 7.0 coincident with that of highly concentrated norfloxacin and that at a low pH corresponds to the absorption pattern at a low concentration, indicating that self-association of norfloxacin occurs at a neutral pH (Figs. 1 and 2). Furthermore, it can be assumed from the existence of the isosbestic points that the system consists of two species at neutral pH—monomer and dimer; consequently, a higher order self-association can be disregarded. The difference in the absorption spectrum between the monomer and dimer is probably due to perturbation between the polycyclic part of the neutral norfloxacin. This assumption is based on the facts that (1) norfloxacin exhibits a light-absorbing pattern similar to that of a monomer in ethanol and a dimer in water (Fig. 3.) and (2) the observation that increasing the urea concentration did not alter the absorption spectrum of norfloxacin (data not shown). The polarity of ethanol is lower than water and therefore inhibits the interaction of π electrons between the stacked norfloxacin.

A molecular modeling study^{20,21} analyzing possible stacking orientations for the enantiomers of neutral ofloxacin (a member of quinolone, with a molecular structure similar to norfloxacin) reported that the shortest distance between two molecular plane was 3.5 Å, this is the distance of the π - π interaction, when stacking occurs with both the piperazine and oxazine ring toward the outside of the stacked plane and the plane of the piperazine ring perpendicular to the plane of the polycyclic part of the ofloxacin molecule.

Binding species of norfloxacin to polynucleotides. It is reasonable to assume that four forms of norfloxacin co-exist in the aqueous solution at neutral pH— a mono-cationic, a neutral (and its zwitter ion), and a mono-anionic species (which would not bind to DNA, therefore it will not be discussed). The dimer is probably formed through stacking of the aromatic part of the norfloxacin. The absorption spectrum and pH titration indicates that the population of the mono-protonated monomer clearly increases when the pH is lowered. Correlating this observation and the fact that the equilibrium constant for the norfloxacin-DNA complex formation increases as pH is lowered, we can conclude that the DNA binding norfloxacin species is a cationic monomer in which the

nitrogen at the end of the piperazine ring is probably protonated (Scheme 1).

We know that the cytosine base is protonated at pH 4.0. Abnormal behavior in the pH dependence of the equilibrium constant may be explained by this factor. When the cationic norfloxacin molecule interacts with the GC region of DNA, the repulsion between the positive charge of norfloxacin and the cytosine disturbs the complex formation, resulting in a lowering of the equilibrium constant. The norfloxacin must bind selectively to the GC base pairs for this effect to be significant. In fact, we observed in the previous study⁷ that the equilibrium constants of norfloxacin-poly[d(G-C)₂] and poly(dG)₂(dC) were larger ($K_{SV}=6.0\sim 7.0\times 10^3\text{ M}^{-1}$) than the norfloxacin complexation with DNA ($K_{SV}=2.8\times 10^3\text{ M}^{-1}$), poly[d(A-T)₂] and poly(dA)₂(dT) ($K_{SV}=0.6\sim 0.7\times 10^3\text{ M}^{-1}$), indicating that norfloxacin binds to the GC rich region of DNA.

CONCLUSION

We concluded that the cationic form of norfloxacin present under acidic condition is the DNA-binding species. Norfloxacin forms dimers at neutral to basic conditions but are monomeric in acidic solutions.

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