Notes

Mechanism of DNA Gyrase Inhibition by Quinolones: I. Spectral Analysis for Nalidixic Acid Polymorphism

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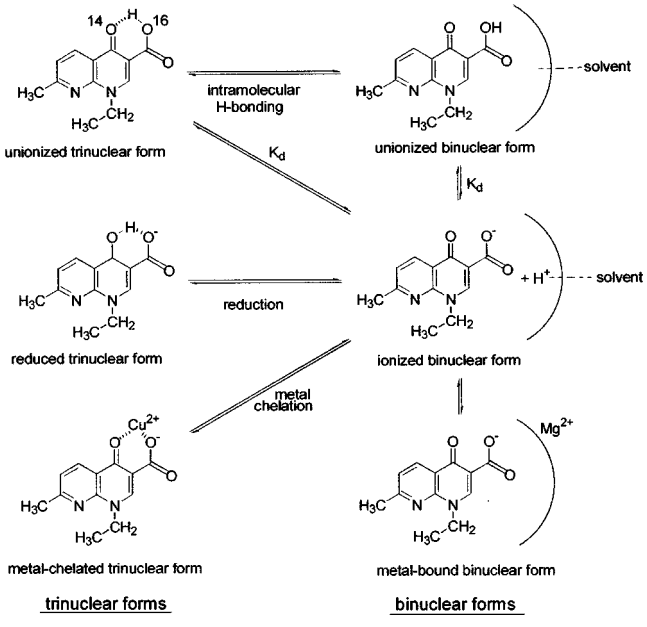
Quinolones such as nalidixic are known to inhibit the action of bacterial type II topoisomerases (e.g., gyrase or topoisomerase IV) that alter the topological state of supercoiled DNA by a series of decatenation and catenation reaction through a transient double-stranded DNA break.[1] however, relatively little is known about the mechanism by which quinolones stimulate DNA cleavage or block their overall catalytic activities.[2] Although early studies[3] focused mainly on DNA gyrase rather than DNA as the potential target of quinolones because of strong electrostatic repulsion between the anionic drug and highly negatively charged DNA at physiological pH, they failed to find the binding site of the drugs. Recently, it was proposed that quinolones bind preferentially to single-stranded DNA region that is revealed when gyrase cleaves DNA during turnover.[4] and the quinolone-stabilized conformational state of the DNA-gyrase complex is responsible for the gyrase inhibition indicating that DNA cleavage is the result rather than a pre-requisite of quinolone binding.[5] Furthermore, direct binding of the quinolones to plasmid DNA by Mg^{2+} mediation[6] and to synthetic double-stranded DNA by even without metal ion mediation[7] were also reported.

These conflicting results and the structural properties of nalidixic acid as discussed below led us to consider that lack of attention to the polymorphism of quinolones depending on variable conditions in cells and change of action mode in response to the polymorphism can somehow be ascribable to the confusion in the understanding of the inhibitory action mechanism of quinolones. From the studies of structure-activity relationships,[8] the 4-oxo-3-carboxylic acid moiety has been proved to be essential to exert antibacterial activity. This carboxylic acid group has relatively high pKa value (6~7). This can be interpreted as due to the formation of intramolecular hydrogen-bonding (H-bonding) between the 4-keto oxygen and the 3-carboxylic hydrogen[9] as shown in Scheme 1. And an X-ray crystallographic study also has shown that the unionized nalidixic acid adopts the trinuclear form having planar geometry by the intramolecular H-bonding of the 4-oxo-3-carboxylic acid moiety.[10] In addition, the local microenvironments of cells can influence the possibility of polymorphism of quinolones via participation of cellular factors such as metal ions,[5,11] local polarity of cellular fluid, and pH as well as the action of reductive enzymes. Thus, elucidation of the possible multiple molecular forms



Scheme 1

of quinolones and the variability of structural entities of quinolones depending on variable environmental conditions, that is sought in the present study, will be important for the understanding of the multiple mechanisms of inhibitory action and clarifying confusions concerning the action mechanism of quinolones.

Experimental Section

Examination of the interaction of nalidixic acid with the environmental factors was performed at 25 by UV/VIS spectrophotometric titrations using Uvicon 860 (Kontron) spectrophotometer.

Results and Discussion

Effects of pH on the Multiple Structural Forms of Nalidixic Acid. The distinctive UV absorption band of nalidixic acid ranging between 300 and 350 nm consists of closely overlapped two subpeaks (Figure 1A). In the examination of solvent effects on spectra (Figure 1A), the blue-shift of the right subpeak (λ_L, longer wavelength) between

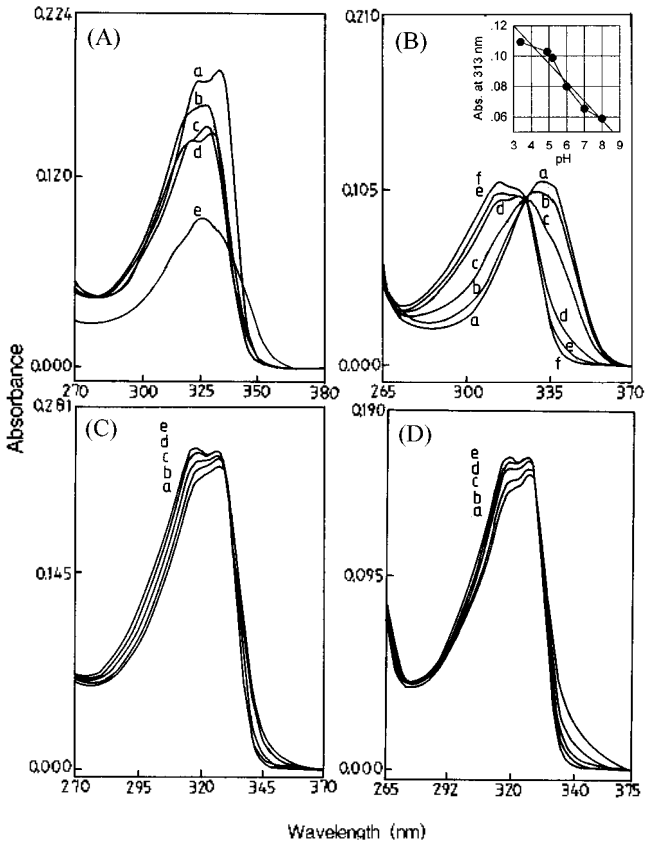


Figure 1. Effects of organic solvents and pH on the nalidixic acid spectra. A: UV absorption spectra were obtained at constant concentratin of nalidixic acid (10 μM) in (a) 1,4-dioxane, (b) methanol, (c) ethanol, (d) N,N,-dimethylformamide, and (e) H_2O. For the pH titration, nalidixic acid solution (10 μM) was titrated with HCl in H_2O (B), methanol (C), and ethanol (D). B: (a) 8.0, (b) 7.0, (c) 6.0, (d) 5.2, (e) 4.9, (f) 3.4. C: (a) 5.3, (b) 5.1, (c) 4.2, (d) 2.9, (e) 0.4. D: (a) 7.9, (b) 7.4, (c), 6.7, (d) 5.3, (e) 2.3.

the two subpeaks is observed in amphiprotic solvents (e.g., methanol and ethanol) in contrast to the case of aprotic solvents (e.g., *N,N*-dimethylformamide and 1,4-dioxane), whereas the left subpeak (λ_S, shorter wavelength) independently to the solvent species stays nearly in the same position. Generally, the n→π* type electronic transitions exhibit the blue-shifts of absorption bands when the solvent is changed from aprotic solvents to polar hydoxylic solvents because of the increased stability of the ground state compared with the excited state of the molecule mainly due to H-bonding of the lone-pair electrons with hydroxylic solvent.[12] Thus, these two subpeaks can be regarded as reflecting an equilibrium of intermolecular H-bonding of nalidixic acid with solvent and intramolecular H-bonding of 4-keto and 3-carboxylic acid groups.

The UV spectrum of nalidixic acid is very susceptible to pH changes as shown in Figure 1B. this pH dependence showed a clear band separation in the wavelength of 300-350 nm into two bands corresponding to the two molecular forms, and λ_{max} was shifted from 332 nm at pH 8 (ionized form) to 313 nm at pH 3.4 (unionized form) with an isosbestic point at 325 nm with decreasing pH. In addition to the equilibrium depending on the dissociation of 3-carboxylic acid, the spectral shapes below pH 6.0 reveal that the other equilibrium arises judging from the appearance of asymmetrical band shapes by the continuous increase of absorption at λ_S. These spectral changes at low pH can be caused by the two factors: First, by the formation of the protonated cationic form at N8 nitrogen (pKa < 5). Second, by the formation of the intramolecular H-bonding of 4-keto and 3-carboxylic acid groups. However, as shown in Figure 1B inset, the profile of absorbance at 313 nm *vs* pH has an inflection point at pH 6.0 reflecting the pKa value of nalidixic acid, but the contribution to the spectral changes of the protonated form at N8 nitrogen which may exist below pH 5 is negligible. Further, the spectroscopic titration was carried out in methanol (Figure 1C) and ethanol (Figure 1D) with varying pH to compare with the data of Figure 1B. In this data, the absorption ratio of λ_S to λ_L ($A_{\lambda S}$ / $A_{\lambda L}$) was gradually increased as decreasing pH in both solvents, which is well consistent with the spectral changes in acidic pH of Figure 1B.

Taken together with the results of solvent effects, these pH-dependent spectral changes may suggest that three structural forms (e.g., unionized trinuclear, unionized binuclear, and ionized binuclear form) of nalidixic acid are in equilibria, and the promotion of the trinuclear-form formation by intramolecular H-bonding upon pH decrease (Scheme 1) as suggested by the increase of absorption at λ_S.

Effects of Prospective Cellular Factors on the Structure of Nalidixic Acid. In terms of molecular mechanism of the drug action of quinolones, the ionized anionic molecules at physiological pH may have difficulty in binding to polyanionic DNA because of electrostatic repulsion. However, if the anionic quinolones form metal complexes with cellular metal ions such as alkali earth metal ions (e.g., Mg^{2+}, Ca^{2+}) or bivalent transition metal ions (e.g., Fe^{2+}, Cu^{2+}, etc.), the binding interactions would become favorable because of increased electrostatic attraction as well as increased hydrophobic interactions. We previously confirmed the formation of 1 : 1 metal chelate complexes of ion-

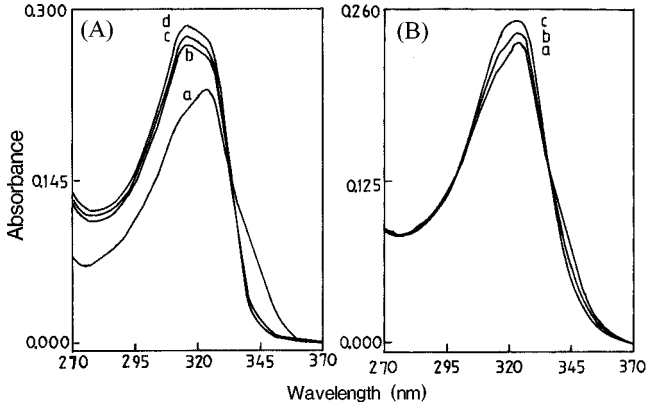


Figure 2. Effects of metal ions on the nalidixic acid spectra. 20 μM of nalidixic acid was titrated with Cu^{2+} (A) and Mg^{2+} (B) in H_2O. The concentrations of Cu^{2+} were (a) zero, (b) 0.1 mM, (c) 0.4 mM, and (d) 1 mM, and of Mg^{2+} are (a) zero, (b) 0.4 mM, and (c) 1 mM.

Table 1. Overlap populations, calculated by EHT MO method, in the ring formation by H-bonding and M^{n+} chelation

M^{n+}	Overlap population	
	M^{n+} O(16)	M^{n+} O(14)
Cu^{2+}	0.3790	0.3578
Mg^{2+}	0.0033	-0.0119

ized quinolones by fluorescence[13] and UV-Vis difference[14] spectroscopic method. Here we compared Mg^{2+}-nalidixate and Cu^{2+}-nalidixate complexes in their spectral differences (Figure 2). When Cu^{2+} was added to the nalidixate solution, the λ_{max} was shifted to 313 nm with overall absorbance increase. In case of Mg^{2+}, however, the absorbance of the nalidixate solution was slightly increased without significant spectral changes upon addition of Mg^{2+}. This data may indicate that the ionized nalidixic acid interacts differently with different metal ions and Cu^{2+}-nalidixate complex can form the trinuclear form by chelation to the 4-oxo-3-carboxylic acid moiety (Scheme 1), and may coincides with our previous data of molecular orbital (MO) overlap-populations, calculated by EHT MO method.[15] in the ring formation by H-bonding and M^{n+} chelation (Table 1).

Solvent polarity of biological fluid at local microenvironments of cells can be an important parameter in binding interaction of quinolones to DNA. As shown in Figure 3, with the decrease of the dielectric constant by increasing 1,4-dioxane content or acetonitrile, $A_{\lambda S}/A_{\lambda L}$ was gradually increased in similar manner to the data of Figure 1C and 1D. Thus, solvent polarity also influences the equilibrium, *viz.*, lower dielectric constants shift the equilibrium to the formation of trinuclear form. In addition, the possibility of the formation of the trinuclear form at physiological pH by enzymatic reduction of 4-keto to 4-hydroxyl group in cells was probed in the presence of reducing agents such as DTT (1,4-dithiothreitol) and ascorbic acid (Figure 4). The spectra

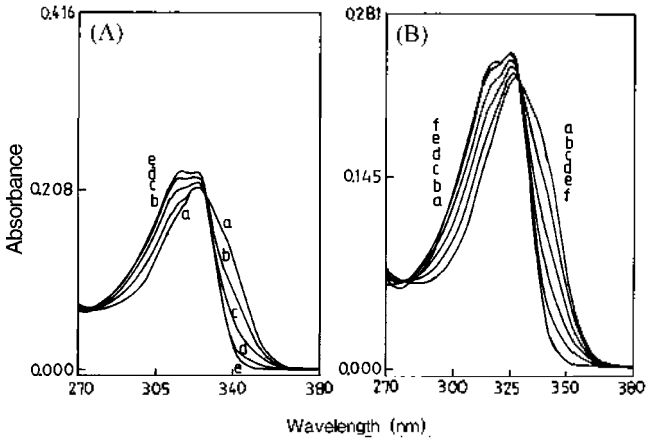


Figure 3. Effects of dielectric constants on the nalidixic acid spectra.. 20 μM of nalidixic acid was titrated with varying concentrations of 1,4-dioxane at pH 6.3 (A) and acetonitrile at pH 7.5 (B) in H_2O. The final concentrations (v/v) of 1,4-dioxane were (a) zero, (b) 20%, (c) 30%, (d) 40% and (e) 50%, and of acetonitrile were (a) 50% (b) 60%, (c) 70%, (d) 80%, (e) 90%, and (f) 100%.

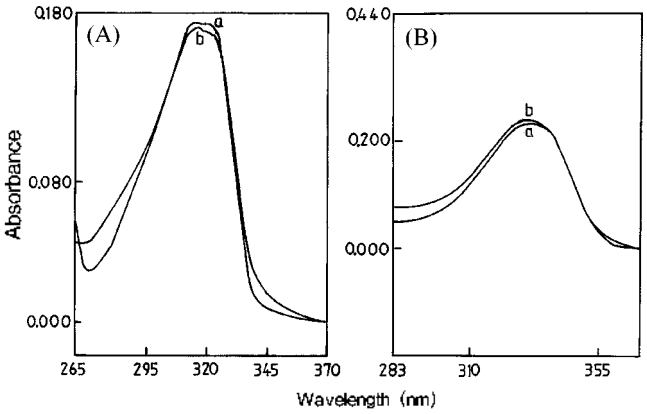


Figure 4. Effects of reducing agents on the nalidixic acid spectra. 20 μM of nalidixic acid was treated with 50 mM of ascorbic acid (A) in 10 mM acetate buffer (pH 5.0) and of DTT (B) in 10 mM Tris buffer (pH 7.5): (a) intact nalidixic acid and (b) nalidixic acid treated with reducing agent.

of nalidixic acid treated with reducing agents have shown positive results by exhibiting the similar characteristic profiles of trinuclear form observed in other cases.

In summary, the data here show that the trinuclear molecular form in equilibrium with the binuclear form can be formed by intramolecular hydrogen bonding of unionized forms at low pH and of reduced molecules even at high pH (e.g., at pH 8.5) and also by chelation of such transition metal ions as Cu^{2+} but not by alkaline earth metal ion such as Mg^{2+}. The formation of the trinuclear molecular form can be favored by organic solvents of lower dielectric constant. From the data, we can infer that various environmental factors may play important roles on the possibility of quinolone polymorphism, which might be related to the multiple mechanism of action of quinolones.

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