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Prototropic and metal complexation equilibria of nalidixic acid in the physiological pH region

W.R. Vincent, S.G. Schulman *, J.M. Midgley **, W.J. van Oort *** and R.H.A. Sorel ***

*College of Pharmacy, University of Florida, Gainesville, FL (U.S.A.); ** School of Pharmacy (Brunswick Sq.), University of London, London (U.K.); and *** Farmaceutisch Laboratorium, Rijksuniversiteit Utrecht, Utrecht (The Netherlands)*

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Summary

The dissociation of nalidixic acid in the near neutral pH region is shown to be due to a single prototropic equilibrium involving the carboxyl group exclusively. Precise dissociation constants as well as stability constants for the binding of the nalidixate anion by several divalent metal ions were determined. The magnitudes of the formation constants of the metal nalidixate complexes support physiological significance of the 1 : 1 complexes and the lack of importance of the 2 : 1 complexes.

Introduction

Nalidixic acid (Ia), 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid, is an antibacterial agent which has been extensively used in the treatment of gram-negative urinary tract infections for over 15 years (Gleckman et al., 1979). It is heat-stable but sensitive to photodecomposition and although soluble in polar organic solvents, it is sparingly soluble in water. The major metabolite of nalidixic acid in man and animals is the corresponding 7-hydroxymethyl analog (Ib) which exhibits antibacterial activity nearly identical in spectrum and potency to that of the parent compound (Gross and Cook, 1974). The mode of action of nalidixic acid has been the subject of wide-spread investigation and has recently been reviewed (Crumplin et al., 1980).

* To whom correspondence should be addressed.

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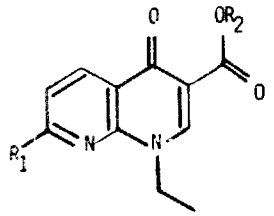
Although the dissociation constant of nalidixic acid in the neutral pH region has been determined by a study of its aqueous solubility (Sulkowska and Staroscik, 1975) and from the pH dependence of its electronic absorption spectrum (Ruzicka et



	R_1	R_2
Ia	$-CH_3$	-H
Ib	$-CH_2OH$	-H
Ic	$-CH_3$	$-C_3H_7$

al., 1975; Timmers and Sternglanz, 1978), it is uncertain which functional group is involved in this equilibrium. The assignment of the dissociation constant to the appropriate functional group of the molecule is fundamental to understanding the physical nature of the drug under physiological conditions and consequently is vital for the discernment of how the drug interacts with metal ions.

Yamabe (1976) has reported that the formation of an iron (II) chelate of nalidixic acid is responsible for the acceleration of the rate of electron transfer from Fe^{2+} to cytochrome c, in vitro. Moreover, a stronger acceleratory effect of some nalidixic acid analogs was correlated with enhanced antibacterial activity. In vitro the activity of nalidixic acid against *E. coli* is potentiated specifically by the presence of the divalent metal ions, Fe^{2+} and/or Cu^{2+} (Crumplin et al., 1980).

The interaction of metal ions with nalidixic acid was first demonstrated by the preparation of the copper and calcium complexes (Lesher and Gruett, 1962). The coordination of nalidixic acid with Fe^{3+} (Dick and Murgu, 1974) and Al^{3+} (Nakano et al., 1978) has also been reported. Stability constants have been determined for 1 : 1 and 2 : 1 (drug : metal) complexes of Fe^{3+} (Ruzicka et al., 1975). Timmers and Sternglanz (1978) have reported stability constants for the formation of 1 : 1 nalidixic acid chelates of Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} ions. The formation of 2 : 1 metal chelates with these metal ions was neglected in this study. In addition, the quantitative evaluation of the stability of the iron (II)–nalidixic acid chelates has not been undertaken, to date.

Despite its antibacterial activity, the hydroxy metabolite of nalidixic acid (Ib) has not been studied in regard to its prototropic reactivity or its metal binding capacity. Since its mode of action is apparently identical to that of nalidixic acid (Staudenbauer, 1976), a comparison of their prototropic dissociation and metal chelate stability constants should assist the understanding of the relationship between specific physicochemical properties and antibacterial activity.

The present study resolves the ambiguity concerning the dissociation constant of nalidixic acid by comparing its spectrophotometric properties with those of the corresponding ester (Ic). In addition, the binding of nalidixic acid and its hydroxylated metabolite to several divalent metal ions of biological significance is investigated.

Materials and methods

Formerly, studies of nalidixic acid have often been limited by the low aqueous solubility of the drug below pH 7 (Sulkowska and Staroscik, 1975). Since moderately intense ultraviolet absorption and fluorescence can be detected from various forms of the drug at concentrations well below its limit of solubility, absorbance and fluorescence spectroscopy were used for quantitation.

Reagents

Sodium hydroxide and perchloric acid were analytical reagent grade from Mallinckrodt Chemical Works. Nalidixic acid was obtained from Fluka. Propyl nalidixate was prepared as previously described (Sorel and Roseboom, 1979). Copper (II) and zinc (II) perchlorate solutions were prepared by the dissolution of metal oxides (Fisher Scientific) by perchloric acid. Cobalt (II) perchlorate was prepared by the addition of perchloric acid to a solution of cobalt (II) acetate (J.T. Baker Chemicals), which was subsequently heated to remove acetic acid. Iron (II) perchlorate was obtained from G. Frederick Smith Chemicals. Iron solutions were prepared and stored under nitrogen. HEPES buffer was from Sigma Chemicals. All analytical dilutions were made using distilled, deionized water.

Instrumentation

Absorption spectra were taken on a Beckman model 25 spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer model MPF-2A spectrofluorometer. All pH measurements were made with an Orion Research model 801 pH meter using a silver–silver chloride glass combination electrode.

The titration data were evaluated utilizing the facilities of the Northeast Regional Data Center of the State University System of Florida located on the campus of the University of Florida in Gainesville employing an Amdahl 470 V/6-11 computer.

Procedures

The pH of all solutions was adjusted by the addition of trace amounts of perchloric acid or sodium hydroxide. The pH of solutions containing the acid or its *n*-propyl ester were adjusted over the range of pH 3–11. The pK_a was calculated from the variation in the longest wavelength absorption band of the conjugate base species as a function of pH, according to the following equation:

$$pK_a = pH + \log\frac{\epsilon_b - \epsilon}{\epsilon - \epsilon_a} \quad (1)$$

where ϵ_a and ϵ_b are the molar absorptivities of the conjugate acid and base species,

respectively, and ϵ is the apparent molar absorptivity for any point in the inflection region of the titration.

Metal ion binding studies were performed by the successive additions of the appropriate quantities of the metal perchlorate solution to a given solution of nalidixic acid in 0.005 M HEPES buffer at pH 7 and 25°C. The fluorescences of the resultant solutions were measured after each addition.

Results and discussion

The change in the absorption of the nalidixic acid as a function of pH corresponds to a calculated pK_a of 6.11 ± 0.02. As shown in Fig. 1, this dissociation may be assigned to the carboxylic acid group (K_{CZ} or K_{NA}), the nitrogen in position 8 (K_{CN} or K_{ZA}) or both in combination considering the possibility of two closely overlapping equilibria.

The absorption spectrum of the propyl ester of nalidixic acid is only slightly different from that of the conjugate acid species of nalidixic acid itself (Fig. 2). The longest wavelength band of the ester absorbs maximally at apparently a slightly longer wavelength. However, this apparent difference may be attributed to different vibrational distributions within the molecules. The peak maximum of the ester corresponds to its lowest energy vibrational feature at 327 nm. The peak maximum of nalidixic acid is 314 nm but the 0–0 vibrational feature of the band occurs as a shoulder at 323 nm which agrees closely with the corresponding band of the ester. The molar absorptivities of the absorption spectra are also similar. Most significantly, the spectrum of the ester was unchanged when recorded at several pH values in the range 3–11. If the ring nitrogen were to undergo deprotonation, a substantial spectral band shift would be expected over this pH range. Therefore, the pK_a may be assigned exclusively to ionization of the 3-carboxylic acid group of the drug, corresponding to equilibrium K_{NA}.

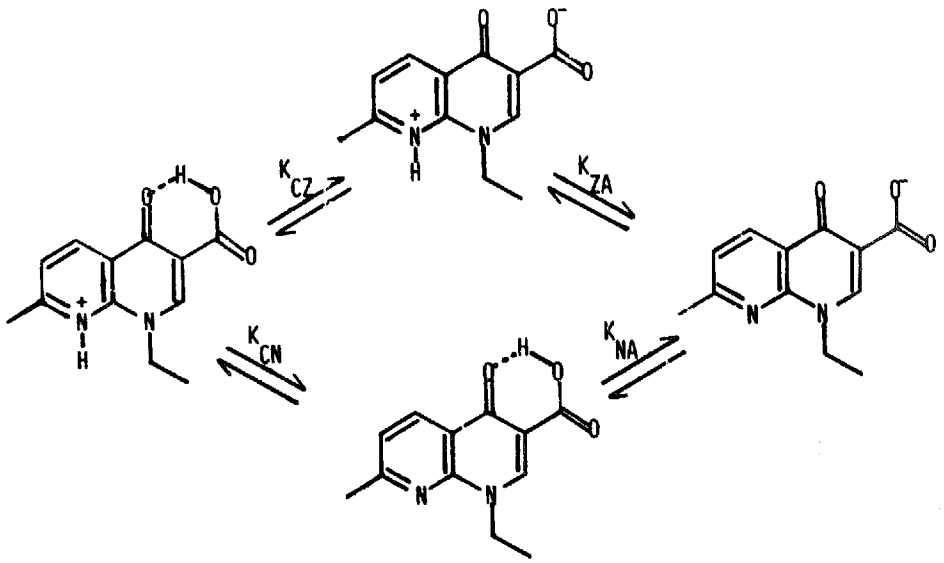


Fig. 1. Possible prototautomeric conformations of nalidixic acid.

The dissociation of the hydroxy metabolite was also examined by the variation of its absorption spectrum with pH and found to be slightly more acidic than nalidixic acid with a calculated pK_a of 5.61 ± 0.02. This may be attributed to a possible hydrogen bond formed between the hydroxymethyl hydrogen and the nitrogen in position 8 which could enhance the inductive electron-withdrawing effect of this nitrogen. This could in turn enhance the acidity of the 3-carboxylic group. It can then be calculated that at physiological pH (7.4.), the percentages of nalidixic acid and of its hydroxy metabolite found in the anionic forms are 95% and 98%, respectively. These findings are in agreement with the results of partition experiments (Sulkowska and Staroscik, 1975) as well as clinical studies which indicate a slightly accelerated elimination of the drug and its metabolite as the urinary pH is elevated (McChesney et al., 1964).

The successive binding of nalidixic acid to divalent metals results in the formation of discrete complexes according to the following reactions:

$$M^{2+} + A^- \rightleftarrows MA^+$$
$$MA^+ + A^- \rightleftarrows MA_2$$

with the stability constants, K_1 and K_2 given, respectively, by:

$$K_1 = \frac{[MA^+]}{[M^{2+}][A^-]} \tag{2}$$

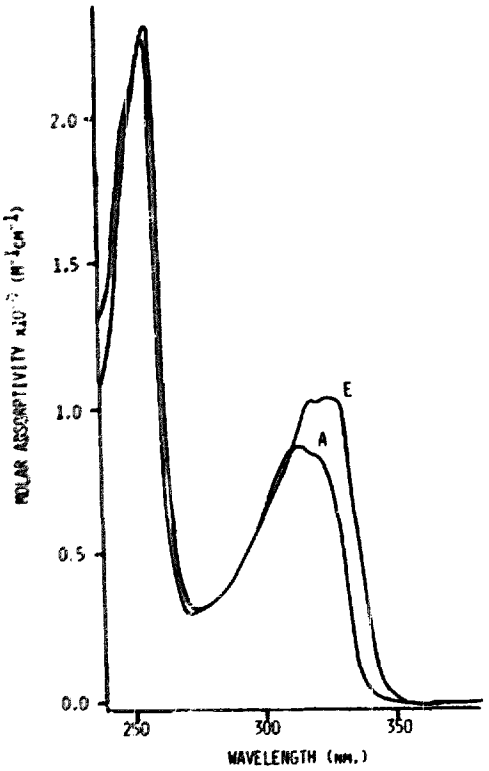
$$K_2 = \frac{[MA_2]}{[MA^+][A^-]} \tag{3}$$

The spectrophotometric determination of stability constants of complexes formed between metals and ligands is dependent on differences between the spectrum of the free ligand and that of its metal complex(es) (Rossotti, 1974). However, the differences between the absorption spectra of nalidixic acid and its metal complexes are quite small (Timmers and Sternglanz, 1978) and so there is a large degree of uncertainty inherent in the determination of stability contants from absorptiometric data.

In contrast, the corresponding fluorescence spectra exhibit large differences. Static quenching is induced by transition metal ions, due to the mixing of the singlet states and low lying states of higher multiplicity by spin-orbit coupling. These states undergo radiationless deactivation by intersystem crossing, making the complexes formed between these metals and nalidixic acid entirely non-fluorescent (Schulman, 1977). Complete quenching of the nalidixate fluorescence was observed in the formation of iron (II), cobalt (II) and copper (II) metal complexes.

However, the fluorescence of nalidixic acid is enhanced approximately four-fold by the complexation with zinc (II), a post-transition metal ion (Fig. 3). The chelation with the metal ion reduces the number of vibrational modes of the nalidixic acid molecule by the formation of a more rigid structure and thus decreases the probability of radiationless deactivation (Schulman, 1977).

The large changes in the intensity of the fluorescence produced by complexation are the basis for the calculation of precise stability constants. The relative fluores-



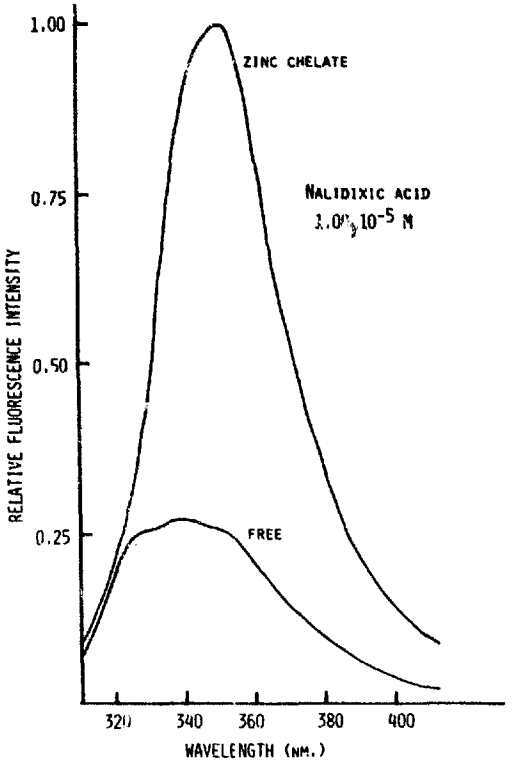


Fig. 2. A comparison of the absorption spectra of nalidixic acid (A) and its propyl ester (E).

Fig. 3. A comparison of the fluorescence spectra of nalidixic acid and its zinc chelate.

cence, F, of a solution containing the acid and a non-quenching metal ion is given by the sum of the individual fluorescent species:

$$F = 2.3\, I_0\phi_A\epsilon_A\ell[A] + 2.3\, I_0\phi_{MA}\epsilon_{MA}\ell[MA] + 2.3\, I_0\phi_{MA_2}\epsilon_{MA_2}\ell[MA_2] \quad (4)$$

and for a transition metal in which the only fluorescent species is the free ligand

$$F = 2.3\, I_0\phi_A\epsilon_A\ell[A] \quad (5)$$

where ϕ_A, ϕ_{MA} and ϕ_{MA_2} are the relative quantum yields of fluorescence and ϵ_A, ϵ_{MA} and ϵ_{MA_2} are the molar absorptivities for the free acid, 1:1 complex and 2:1 complex, respectively, I_0 is the intensity of the exciting light and ℓ is the optical pathlength. These equations combined with Eqns. 2, 3 and the equations for the formal concentrations of acid (6) and metal ion (7):

$$C_A = [A] + [MA] + 2[MA_2] \quad (6)$$

$$C_M = [M] + [MA] + [MA_2] \quad (7)$$

were solved simultaneously by employing a Newton-Raphson non-linear least-squares computer algorithm (Lingane and Hugus, 1970). The variable parameters for the least-squares routine were the stability constants and, in the case of the zinc complexes, the products $\phi_{MA}\epsilon_{MA}$ and $\phi_{MA_2}\epsilon_{MA_2}$. The resulting stability constants for

TABLE I

Stability constants for the complexation of divalent metal ions by nalidixic acid and its hydroxylated metabolite

Ligand	Metal ion	log K_1^a	log K_2^a	log K_1^b
Nalidixic Acid	Fe^{2+}	3.86±0.03	3.0±0.1	–
	Co^{2+}	4.22±0.02	3.0±0.1	4.4
	Cu^{2+}	5.38±0.03	4.1±0.1	5.5, 5.6
	Zn^{2+}	3.30±0.03	2.3±0.1	3.8
Hydroxynalidixic Acid	Fe^{2+}	3.79±0.03	2.9±0.1	–
	Co^{2+}	3.98±0.02	3.1±0.1	–
	Cu^{2+}	5.44±0.03	4.0±0.1	–
	Zn^{2+}	3.33±0.03	2.5±0.1	–

[a] Present work; [b] Timmers and Sternglanz (1978).

the 1 : 1 and 2 : 1 complexes of nalidixic acid and the hydroxy metabolite with cobalt (II), iron (II) and zinc (II) are given in Table I.

The stability constants obtained for complexes with a ligand to metal ion ratio of 1 : 1 are in general agreement with the previous values of Timmers and Sternglanz (1978) for cobalt (II), copper (II) and zinc (II) which are also shown in Table I. The values reported here are precise due to the increased sensitivity of the fluorescence technique. The stability constant for the 1 : 1 iron (ii) complex of nalidixic acid has been determined for the first time. These stability constants are of sufficient magnitude to suggest that nalidixic acid may be associated with trace metal ions in vivo. By comparison, the stability constants determined for complexes with a 2 : 1 ligand to metal ratio were found to be significantly smaller, indicating that only a minor fraction of the drug will form such complexes under physiological conditions. In this case, it is apparent that the insolubilities of the neutral 2 : 1 complexes would not remove much of the nalidixate or trace metal ions from the physiological environment.

The similarities of the metal chelate binding constants for nalidixic acid and its hydroxy metabolite coupled with the similarities between their antibacterial activity reinforce the previous hypothesis that the mode of action of each of these two compounds is through a common metal chelate mechanism.

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