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Research paper

Protonation equilibrium and lipophilicity of olamufloxacin (HSR-903), a newly synthesized fluoroquinolone antibacterial

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Abstract

This study was performed to characterize the protonation equilibrium at the molecular level and pH-dependent lipophilicity of olamufloxacin. The deprotonation fraction of the carboxyl group as a function of pH was specifically calculated at the critical wavelength 294 nm, where UV pH-dependent absorbance of olamufloxacin was independent of the ionized state of the aminopyrrolidinyl amino group but heavily depended on that of the carboxyl moiety. Accordingly, micro-protonation equilibrium could be described using a nonlinear least-squares regression program MULTI. In contrast, macro-protonation equilibrium was depicted at most wavelengths where olamufloxacin absorbance was influenced by ionized states of both proton-binding groups, results coinciding with the former. Furthermore, distribution features of four microspecies in aqueous phase were assessed. The apparent partition coefficient versus pH profile of olamufloxacin showed a parabolic curve in *n*-octanol/buffer system which reached peak near pH 8, agreeing with the above determined isoelectric point (p*I*). Ion-pair effect was observed for olamufloxacin under an acidic condition, eliciting experimental values higher than those theoretically calculated, which was similar to ciprofloxacin but not levofloxacin due to amino group type. Moreover, olamufloxacin was moderately lipophilic in comparison with other quinolones, with an apparent partition coefficient of 1.95 at pH 7.4.
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Keywords: Olamufloxacin; Protonation equilibrium; Micro-dissociation constant; Apparent partition coefficient; Ion pairing

1. Introduction

Olamufloxacin (HSR-903) has recently been investigated in clinical trials as an oral, novel fluoroquinolone. It has the 6-fluorine substituent generally present in the currently used quinolones and contains an amino group at position 5, an aminopyrrolidinyl group at position 7 and a methyl group at position 8 (Fig. 1). It has been shown that olamufloxacin possesses a more potent antibacterial activity against potential respiratory pathogens compared with other quinolone derivatives [1,2]. In addition, it distributes well into tissues, such as those with a low fat content like lung and liver, but not the exception of brain, and exhibits low toxicity [3–5]. However, very few research has been carried out on its biologically important physicochemical parameters, for example, pK_a and lipophilicity, which exert a key role in determining its absorption, transport, and receptor binding at a molecular level.

Unusually, for amphoteric quinolones such as olamufloxacin, its chemical structure contains two proton-binding sites with similar basicity, namely the aminopyrrolidinyl amino and carboxyl groups, and deprotonation of the two proton-binding groups overlaps to a certain extent. Accordingly, there are four microspecies for the amphoteric quinolone in aqueous solution, namely positive, zwitterionic, neutral and negative microforms at the molecular level [6]. An accurate description of the distribution of these microspecies is of great significance in understanding drug-biomembrane interactions, drug-receptor associations, and, thus, biological actions, all of which require both the drug molecules and receptor surface being presented in the appropriate complementary conformations.

In this study, we determined the acid-base properties of olamufloxacin at the molecular level in terms of macro- and

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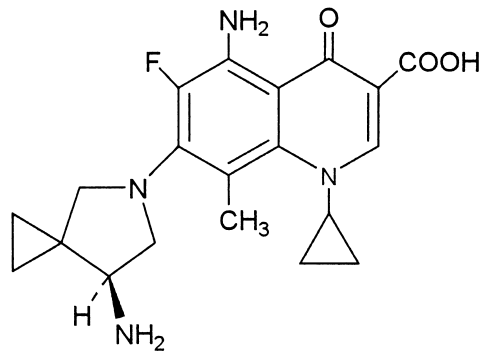

Fig. 1. Chemical structure of olamufloxacin.

micro-dissociation constants by means of a spectrophotometric technique, and studied its pH-dependent behavior in terms of the apparent partition coefficient in an *n*-octanol/buffer system.

2. Materials and methods

2.1. Materials

Olamufloxacin and NQ-762 (internal standard for HPLC assay) were synthesized and provided by Hokuriku Seiyaku Co., Ltd. (Fukui, Japan). An *n*-octanol was purchased from Sigma Chem. Co. (St. Louis, MO, USA) and all other reagents were of at least analytical grade.

2.2. Spectrophotometric measurement of protonation equilibrium

Three aliquots of 40 μM olamufloxacin solution were prepared in 0.03 M NaH_2PO_4, 0.03 M Na_2HPO_4, or 0.03 M Na_3PO_4, with a total ionic strength of 0.2 M using NaCl. Olamufloxacin solutions of different designated pH values, ranging from 3.5 to 12.5, were prepared by mixing three stock solutions. The absorbance versus wavelength of the resultant solutions was determined and recorded in a spectrophotometer (DU-650, Beckman Instrument Inc., Fullerton, CA, USA). Each determination was performed in duplicate at 25 °C.

2.3. Determination of apparent partition coefficient

The apparent partition coefficient in an *n*-octanol/buffer system ($D_{O/B,pH}$) was measured by the shake-flask technique. Blank phosphate buffer solutions of predetermined pH were prepared as described above and pre-saturated with *n*-octanol. Equal volumes of the aqueous and organic phases (pre-saturated with appropriate phosphate buffer solutions) were mixed, olamufloxacin stock solution (2 mM) added to give a final aqueous concentration of 10 μM, and then shaken in a thermostatically controlled water incubator at 25 °C for 4 h. A pilot study had shown that this time allowed the partitioning equilibrium to be reached. Then, samples were centrifuged ($700 \times g$) and the two phases were separated. The olamufloxacin concentration in the aqueous phase was determined by HPLC and that in the *n*-octanol phase was calculated in terms of mass balance. The reported values were the average of three parallel measurements and the 95% confidence interval associated with each value was not more than 0.05.

2.4. HPLC analysis

The HPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-10AD pump, LC-10AD ultraviolet detector and C-R6A data process integrator, and a reverse phase RP-18 column (5 μm, 4.0×150 mm, Kanto Chem. Co., Tokyo, Japan) was used. The mobile phase consisted of 0.03 M ammonium phosphate buffer (pH 2.5) and acetonitrile (3:1, v/v). The flow rate was 1.1 ml/min and the column temperature was maintained at 35 °C. The eluate was monitored at 308 nm.

2.5. Curve-fitting calculation

The curve-fitting calculation to obtain the 'best' microscopic and macroscopic constants as judged by the least squares criterion was performed with the program MULTI [7]. The input data were weighted as the reciprocal of the observed values and the Damping Gauss Newton method was used as the fitting algorithm.

3. Results and discussion

3.1. Evaluation of protonation equilibrium for olamufloxacin

Quinolone molecules contain two proton-binding sites with similar basicity (carboxyl and aminopyrrolidinyl amino groups of olamufloxacin) and consequently exist as four micro-protonation microforms in aqueous solution. The micro-dissociation constant characterizes the basicity of the individual proton-binding site while the macro-dissociation constant cannot be assigned to a specific group [8,9]. The protonation equilibrium of olamufloxacin is shown in Fig. 2.

The micro-dissociation constants (k_1, k_2, k_{12}, k_{21}) are defined as

$$k_1 = [\mathrm{II}][\mathrm{H}^+]/[\mathrm{I}] \quad (1)$$

$$k_2 = [\mathrm{III}][\mathrm{H}^+]/[\mathrm{I}] \quad (2)$$

$$k_{12} = [\mathrm{IV}][\mathrm{H}^+]/[\mathrm{II}] \quad (3)$$

$$k_{21} = [\mathrm{IV}][\mathrm{H}^+]/[\mathrm{III}] \quad (4)$$

where I, II, III, and IV denote the positive, zwitterionic, neutral and negative microspecies, respectively.

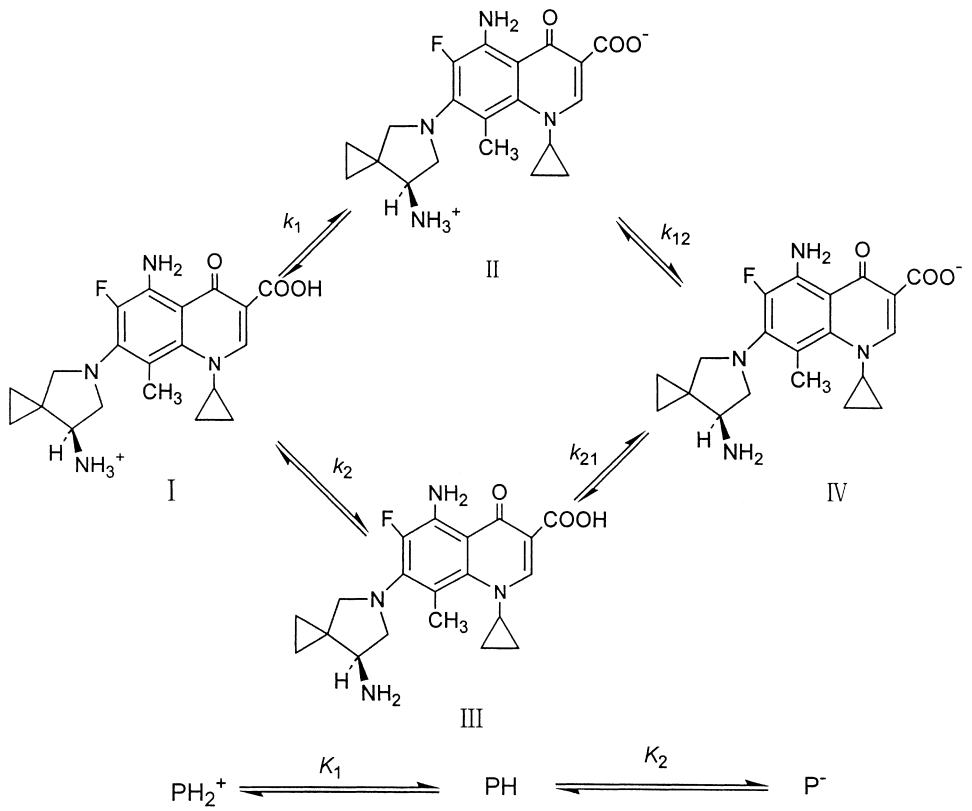

Fig. 2. Protonation equilibrium of olamufloxacin. I, II, III, and IV represent the positive, zwitterionic, neutral and negative microspecies, k_1, k_2, k_{12}, k_{21} denote the micro-dissociation constants of the individual functional group, K_1 and K_2 represent stepwise macro-dissociation constants, respectively. PH_2^+ represents the cationic species, PH represents the zwitterionic species including zwitterionic and neutral microspecies, and P^- represents the anionic species.

The macro-dissociation constants (K_1, K_2) are expressed as

$$K_1 = \frac{[PH][H^+]}{[PH^{2+}]} = \frac{([II] + [III])[H^+]}{[I]} \quad (5)$$

$$K_2 = \frac{[P^-][H^+]}{[PH]} = \frac{[IV][H^+]}{[II] + [III]} \quad (6)$$

where PH_2^+ represents the cationic species equivalent to the positive microspecies, PH represents the zwitterionic species including the zwitterionic and neutral microspecies, and P^- represents the anionic species equivalent to the negative microspecies, respectively.

The macro-dissociation constants (K_1, K_2) have a clear relationship to the micro-dissociation constants

$$K_1 = k_1 + k_2 \quad (7)$$

$$1/K_2 = 1/k_{12} + 1/k_{21} \quad (8)$$

$$K_1 K_2 = k_1 k_{12} = k_2 k_{21} \quad (9)$$

It is reasonable to conclude that only three of the six micro- and macro-dissociation constants are independent as a result of the above relationships.

A spectrophotometric technique for determining the microscopic constants requires that one group in the molecule gives rise to no interfering absorption in either the acidic or conjugated basic form of the other group [10]. Therefore, the deprotonation fraction of one of the two proton-binding groups as a function of pH can be specifically determined from the UV absorption spectra. In the case of quinolones, the prerequisite is that the pH-dependent UV absorption spectra are independent of the protonation state of the piperazinyl moiety, but are directly dependent on that of the carboxyl group [6]. So, the molar extinction coefficients of the two protonated carboxyl group forms I and III, and the two deprotonated carboxyl group forms II and IV, respectively, are identical at the analyzing wavelengths.

Both the UV absorbance versus wavelength (A) and versus pH value (B) profiles of olamufloxacin are illustrated in Fig. 3. Evidently there is an isosbestic point for olamufloxacin at 255 nm from both plots. Since the absorbances are identical over the entire pH range, the molar extinction coefficients of I, IV, and the averaged molar extinction coefficient of II and III are equivalent at 255 nm. Moreover, there is probably a different molar extinction coefficient for the individual microspecies of II and III, because it is unclear whether the ionized state of the aminopyrrolidinyl amino group has an effect on the UV absorption spectra of olamufloxacin.

As far as the absorbance-pH curves were concerned, the UV absorption spectra of olamufloxacin were affected by the ionized state of both the carboxyl and aminopyrrolidinyl amino groups at most wavelengths (Fig. 3). This is

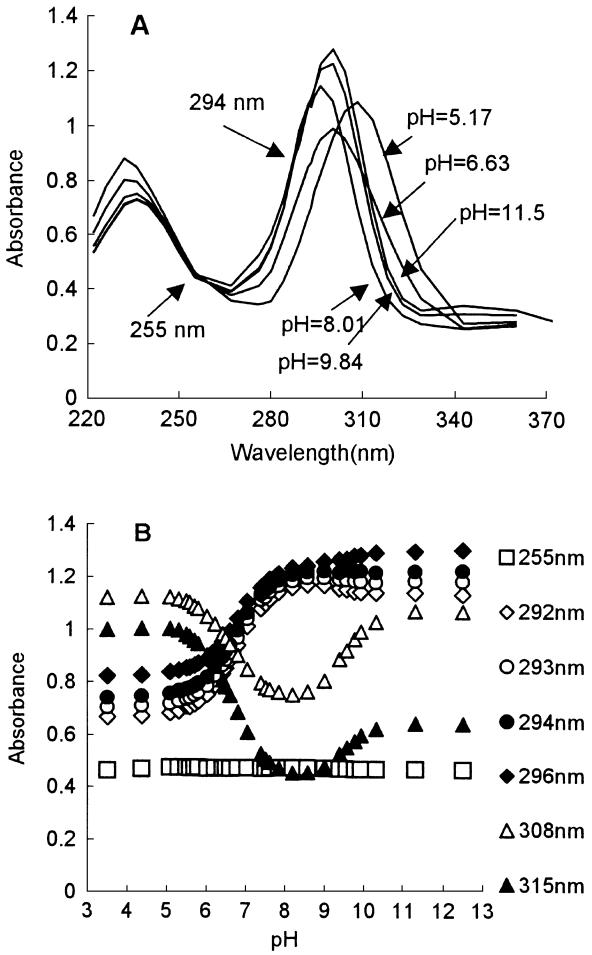

Fig. 3. pH-Dependent UV absorption spectra for olamufloxacin. (A) Absorbance–wavelength and (B) absorbance–pH profiles.

markedly different from the situations with ciprofloxacin [11]. Therefore, the molar extinction coefficients of I and III, or II and IV, are not identical, respectively. Then, the prerequisite of the above analysis for microscopic protonation equilibrium isn't fulfilled by olamufloxacin. Accordingly, the fractional deprotonation of one of the two proton-binding groups couldn't be selectively and specifically determined from the UV pH-dependent absorption spectra of olamufloxacin.

By contrast, taking the wavelength of 294 nm into account, the pH-dependent UV absorbance of olamufloxacin was markedly dependent on the protonation state of the carboxyl moiety but independent of the ionized state of the aminopyrrolidinyl group (Fig. 3). At this critical wavelength, the molar extinction coefficient of I and III, and II and IV, were identical, respectively. Thus the deprotonation fraction of the carboxyl group as a function of pH could be specifically acquired. On either side of this wavelength, the protonation state of the aminopyrrolidinyl group probably exerted to a less extent influence on the UV absorbance of olamufloxacin, perhaps due to stereo-conformational intervention (Fig. 3).

On the basis of the olamufloxacin pH-dependent UV absorbance at 294 nm, the deprotonation fraction (α_{coo^-}(pH)) of carboxyl group can be calculated by

$$\alpha_{COO^-(pH)} = \frac{A_{(pH)} - A_{(COOH)}}{A_{(COO^-)} - A_{(COOH)}} \quad (10)$$

where $A_{(coo^-)}$ and $A_{(cooH)}$ are experimental absorbance values at extremely basic and acidic conditions, respectively, and α_{coo^-}(pH) is the deprotonation fraction for the carboxyl moiety at 294 nm.

Based on Fig. 2, the deprotonation fraction of the carboxyl group (α_{coo^-}(pH)) can also be expressed as

$$\alpha_{COO^-(pH)} = \frac{[II] + [IV]}{[I] + [II] + [III] + [IV]}$$

$$= \frac{k_1[H^+] + K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2} \quad (11)$$

The macro- and micro-dissociation constants (pK_1, pK_2 and pk_1) were estimated from the deprotonation fraction (α_{coo^-}(pH)) as a function of the pH data by an iterative nonlinear least squares analysis in terms of Eq. (11) using the MULTI program [7]. Shown in the Fig. 4, were the solid line, α_{coo^-}(pH) versus pH curves generated from the determined constants, which agreed well with the experimental values for olamufloxacin. Other micro-dissociation constants (pk_2, pk_{12}, pk_{21}) were then calculated from pK_1, pK_2 and pk_1 according to Eqs. (7)–(9) (Table 1). As the macro- and micro-dissociation constants of olamufloxacin have not been reported previously, the resultant parameters could not be compared.

The macro-dissociation constants of pK_1 and pK_2 largely reflect the basicity of the carboxyl and aminopyrrolidinyl amino groups, respectively, for the following reasons.

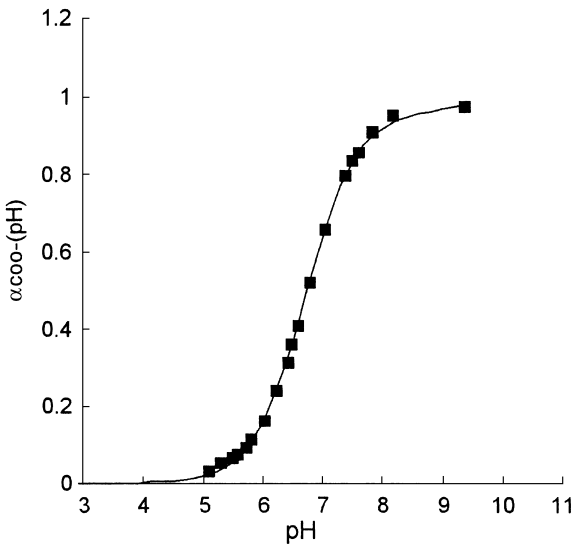

Fig. 4. (α_{coo^-}(pH))-pH profiles for olamufloxacin. The deprotonation fraction of carboxyl group, α_{coo^-}(pH), was determined at 294 nm, and the solid line shows the computer-generated simulation curves using the determined dissociation constants.

Table 1
Macro- and micro-dissociation constants for olamufloxacin

Method	pK_1	pK_2	pk_1	pk_2	pk_{12}	pk_{21}	pI
A[a]	6.71	9.45	6.73	8.10	9.43	8.06	8.08
B[b]	6.72	9.41					8.07

[a] The micro- and macro-dissociation constants were determined by fitting pH-dependent α_{COO^-}(pH) data at 294 nm.
[b] The macro-dissociation constants were determined by fitting pH-dependent A_T data at 308, 313, and 318 nm, respectively, and are expressed as the average.

Firstly, the electron-attracting effect of the aromatic ring diminishes the basicity of the aminopyrrolidinyl N_1' atom and the amino group at position 5; secondly, the quinoline ring nitrogen has little appreciable basicity in aqueous solution due to the larger conjugated electron cloud in the aromatic ring [6]. The pK_1 (6.71) of olamufloxacin was slightly higher than that of ciprofloxacin (6.09) [11], perhaps owing to the different strengths of the electron-donating effect of methyl and amino substituents in the quinoline ring. Moreover, it was much higher than lomefloxacin (5.49) probably because of the strong electron-attracting effect of the fluorine substituent in lomefloxacin [6]. Furthermore, these compounds were much weaker acids than aromatic carboxylic acids. The reduced acidity may be ascribed to the formation of an intra-molecular hydrogen bond between the carboxyl and neighboring keto groups in the quinoline ring, resulting in stabilization of the protonated form of the carboxyl group. The pK_2 of olamufloxacin (9.45) was 0.65 pH unit greater than that of ciprofloxacin (8.74) [11]. The structural differences may account for the above discrepancies because of the types of amino group, bicyclical structure and stereo-conformation of olamufloxacin.

Based on the macro- and micro-dissociation constants, the distribution-pH profiles of the four microspecies in solution could be characterized in detail [11]. Distribution diagrams of the four microspecies of olamufloxacin are shown in Fig. 5. It is clear that the zwitterionic microform predominates over the neutral microspecies and both reach a maximum at pH around an isoelectric point (pI, 8.08). The ratio of the neutral to zwitterionic microspecies equivalent to k_2/k_1, was identical irrespective of the pH and was one of the unique characteristics of the individual quinolone antibacterial. It was 4.24% for olamufloxacin, over two-fold higher than ciprofloxacin (2.09%) but almost two-fold lower than grepafloxacin (7.64%) [11]. It has been found that this ratio differs considerably among quinolone antibacterials, heavily influencing their lipophilicity and thus determining their ability to reach the interior of bacteria [6,11].

3.2. Determination of macro-dissociation constants for olamufloxacin

Since the UV absorption spectra of olamufloxacin are affected by the ionized state of both the carboxyl and aminopyrrolidinyl amino groups at most wavelengths, an alternative method of determining the macro-dissociation constants is described.

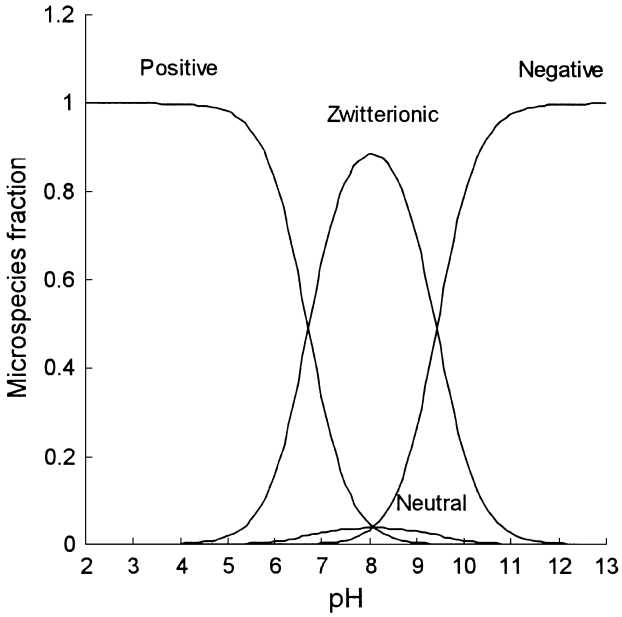

Fig. 5. Distribution of four microspecies for olamufloxacin in aqueous phase.

At the macroscopic level, olamufloxacin molecules are ionized over the whole pH range and exist in three species, namely, cation, zwitterion and anion. As the apparent absorbance of the tested solutions is the sum of the absorbance of all the individual species, the relationship between the apparent absorbance and pH is defined as

$$A_T = \frac{A_C[H^+]^2 + A_Z K_1[H^+] + A_A K_1 K_2}{[H^+]^2 + K_1[H^+] + K_1 K_2} \quad (12)$$

where A_T represents the apparent absorbance of the test solution, A_C and A_A represent the individual absorbance of the cationic and anionic species, corresponding to the experimental absorbance values under extremely acidic and basic conditions, respectively, and A_Z represents the absorbance of the zwitterionic species.

The macro-dissociation constants (pK_1 and pK_2) were then calculated by fitting the apparent absorbance data versus pH values according to Eq. (12) using the MULTI program at several wavelengths (308, 313, and 315 nm) and the mean values obtained (Table 1). A plot of absorbance versus pH at 308 nm simulated by the estimated constants is shown in Fig. 6, illustrating the good agreement between the experimental and simulated values. In comparison with the former method, the macro-dissociation constants obtained by both approaches are in good agreement with each other, implying the appropriateness of determining the olamufloxacin microscopic protonation equilibrium using the critical wavelength (294 nm) in the UV pH-dependent absorption spectra.

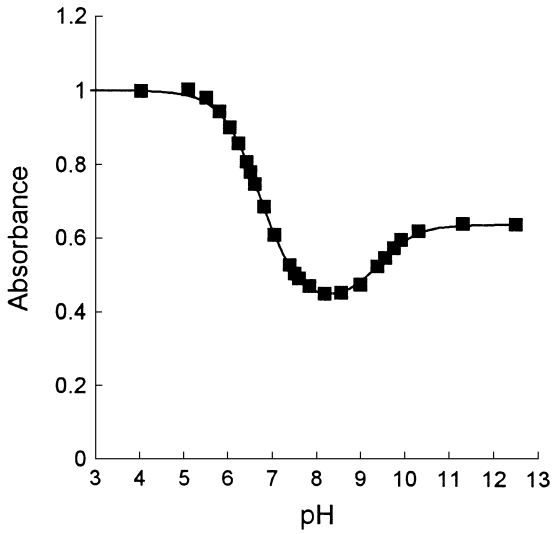

Fig. 6. Apparent absorbance-pH for olamufloxacin at 308 nm. Solid line represents the computer-generated simulation curves using the estimated constants.

3.3. pH-Dependence of the apparent partition coefficient for olamufloxacin

Fig. 7 shows the parabolic curve of the pH-dependent apparent partition coefficient for olamufloxacin in an *n*-octanol/buffer system. The apparent partition coefficient (log $D_{O/B,pH}$) reached a maximum at pH 8.0 for olamufloxacin, which coincided with the above estimated p*I* value (8.08). Moreover, olamufloxacin is moderately lipophilic, as far as the quinolone antibacterials are concerned, with a $D_{O/B,7.4}$ of 1.95, compared with grepafloxacin (4.60) and ciprofloxacin (0.08) [11].

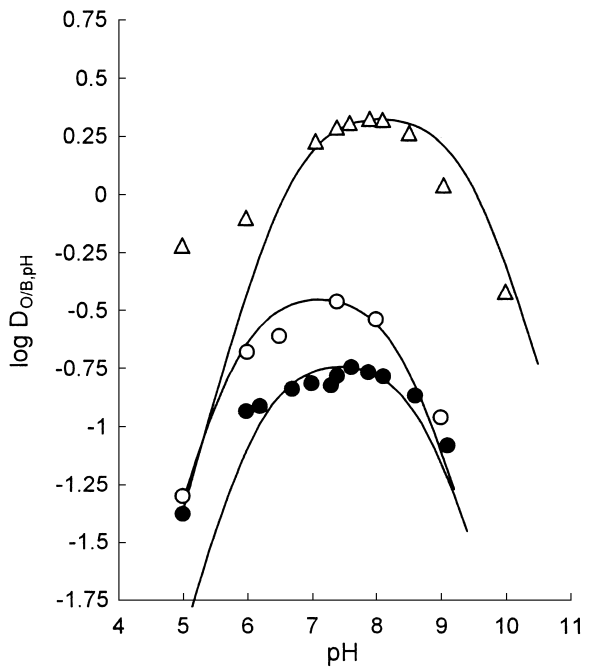

Fig. 7. Apparent partition coefficient of olamufloxacin in an *n*-octanol/-buffer system. Solid lines represent the calculated log $D_{O/B,pH}$ based on the estimated log $P_{O/B}$ value of olamufloxacin (Δ), ciprofloxacin (●)[11], levofloxacin (○) [Unpublished data].

The thermodynamic partition coefficient is defined by the law of distribution

$$P_{O/B} = [III]_o/[III]_w \quad (13)$$

where $[III]_o$ and $[III]_w$ represent the concentrations of neutral microspecies in organic and aqueous phases, respectively.

Takacs-Novak et al. [12]have confirmed by a spectrophotometry that only the neutral microspecies is capable of partitioning into the organic *n*-octanol phase and thus

$$\begin{aligned} D_{O/B,pH} &= [III]_o/([I]_w + [II]_w + [III]_w + [IV]_w) \\ &= ([III]_o/[III]_w)([III]_w/([I]_w + [II]_w \\ &\quad + [III]_w + [IV]_w)) \\ &= P_{O/B} \cdot f(III) \end{aligned} \quad (14)$$

$$P_{O/B} = D_{O/B,pH}/f(III) \quad (15)$$

where *f*(III) represents the distribution fraction of the neutral microspecies.

It has been found that some quinolone antibacterials can form an ion-pair between their positively charged microspecies and the negatively charged ions of buffer under acidic conditions, accordingly leading to deviations from their expected partitioning behavior at lower pH values [13]. Moreover, the ability of forming an ion-pair is as follows: primary > secondary > tertiary amino group [14]. Since olamufloxacin contains a primary amino group, it possesses a relatively higher potential to form an ion-pair which can partition into the *n*-octanol phase. Therefore, the log $P_{O/B}$ of olamufloxacin was calculated under neutral conditions to be 1.75 in order to avoid the ion pairing effect. The solid line, log $D_{O/B,pH}$ versus pH curves generated using the estimated $P_{O/B}$ value and determined dissociation constants, approximately agreed with the experimental values for olamufloxacin under neutral and basic conditions in Fig. 7. However, under an acidic condition, a marked deviation from the predicted values was observed, perhaps due to the ion-pairing effect, consistent with that seen with ciprofloxacin [11], but not levofloxacin [Unpublished data]. This result clearly shows that the type of amino group in quinolones exerts a significant effect on the partitioning into the *n*-octanol phase under an acidic condition.

In conclusion, taking the unique characteristics of the olamufloxacin UV pH-dependent spectra into consideration, either the micro-dissociation or macro-dissociation constants can be determined under the special analysis conditions required. In addition, zwitterionic microspecies always predominated over neutral protonation isomers, and the concentration ratio of the neutral to zwitterionic microforms was markedly different from one quinolone derivative to another. The apparent partition coefficient

versus pH profiles of olamufloxacin showed a maximum, and its shape deviated from the expected one as a result of the ion-pair effect due to its amino group type. olamufloxacin was moderately lipophilic compared with other quinolone antibacterials, with $D_{O/B,7.4}$ of 1.95.

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