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Physicochemical properties of enrofloxacin

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Abstract

The physicochemical properties of enrofloxacin, a fluoroquinolone that inhibits the activity of bacterial DNA gyrase, are described. Its spectral, solubility and related physicochemical characteristics are discussed. The dissociation behaviour of enrofloxacin was examined by UV spectrophotometry at 25°C in a series of buffers ranging from pH 1 to 10. The corresponding macro- and microscopic dissociation constants were calculated. The apparent *n*-octanol–water partition coefficients were measured from pH 2 to 10. © 1997 Elsevier Science B.V.

Keywords: Fluoroquinolone; Enrofloxacin; Solubility; Dissociation constants; Partition constants; Physicochemical properties

1. Introduction

In recent years, fluoroquinolones, a group of drugs derived from nalidixic acid, have been developed as high-potency antibacterial agents. These drugs are used in the treatment of diseases caused by intracellular bacteria like *Mycobacterium*, *Mycoplasma*, *Chlamydia*, *Legionella* and *Brucella*. Fluoroquinolones inhibit the activity of bacterial DNA gyrase, an enzyme which controls the supercoiling of DNA by converting relaxed covalently closed circular DNA to a superhelical form by an energy-dependent strand breakage and resealing process [1]. One of these quinolones, enrofloxacin, has shown its efficacy in the treatment of the main bacterial processes affecting farm animals [2]. As these antibiotics do not concentrate effectively in cells that act as hosts for these pathogens, it has been pointed out that encapsulation of quinolones in liposomes could improve their effectiveness [3]. A scaled-up method of loading enrofloxacin in this way has been described elsewhere [4].

Thorough knowledge of the physicochemical and analytical properties of any drug is of paramount importance for successful research and its optimal use. Literature data on the physicochemical properties of some quinolones (for example, nalidixic acid, ciprofloxacin or norfloxacin) are extensive. Nevertheless, the paucity of literature references on the physicochemical properties of enrofloxacin is surprising. This paper attempts to provide full information on some of the most interesting physicochemical properties of this antimicrobial drug.

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2. Spectroscopic properties

2.1. Absorption of UV-radiation

Absorption of UV-radiation was determined in several phases (water, acetate buffer pH 4.7, methanol, ethanol, chloroform). Measurements of absorbance were carried out in a Hitachi U-2000 spectrophotometer (Hitachi, Japan). Table 1 shows the wavelengths in which an absorption peak appears with its corresponding molar absorptivity. From this table it can be inferred that values of maxima are influenced by the polarity of the solvent. Enrofloxacin suffers a slight bathochromic shift as the polarity of the solvent decreases.

However, the absorption of the molecule is pH-dependent. In Fig. 1 the existence of two isosbestic points at 270 ± 1 and 314 ± 2 nm is seen. An increase of pH produces a hypsochromic shift of the highest maximum while the minor one undergoes a bathochromic shift. As an example, the variation of pH from 2.8 to 8.8 involves a change in one maximum from 276 to 270 nm and from 314 to 322 in the other.

Table 1
Molar absorptivity (ε) of enrofloxacin in several solvents at the wavelength of maximal absorption (λ_{max})

Phase	λ_{max}	$\varepsilon(M^{-1} \cdot cm^{-1})$
Water	276	58 500
	318	17 500
Acetate buffer	276	57 400
	318	17 900
Ethanol	280	65 500
	322	20 200
Methanol	280	78 300
	322	22 700
Chloroform	282	54 700
	324	16 500

Values were obtained from a solution of 6 μg ml^{-1} enrofloxacin ($n = 6$).

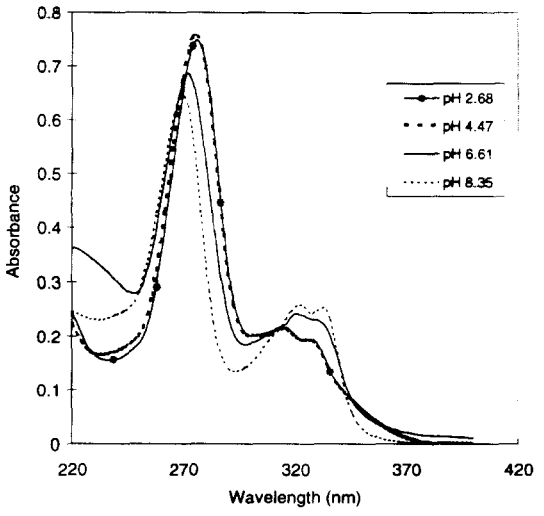

Fig. 1. Spectra of UV absorption of enrofloxacin at 4 pH's

2.2. Fluorescence

Fluorescence measurements were carried out in a fluorescence spectrophotometer Hitachi F-2000 (Hitachi, Japan) with a 10 nm bandpass, fitted with a 150-W xenon arc lamp. Fig. 2 shows the excitation and emission spectra obtained. At pH 6.8 enrofloxacin is excited at 275 and 315 nm. The emission spectrum exhibits a maximum at 439 nm after excitation at 315 nm. This spectrum is similar to that obtained by other authors [5].

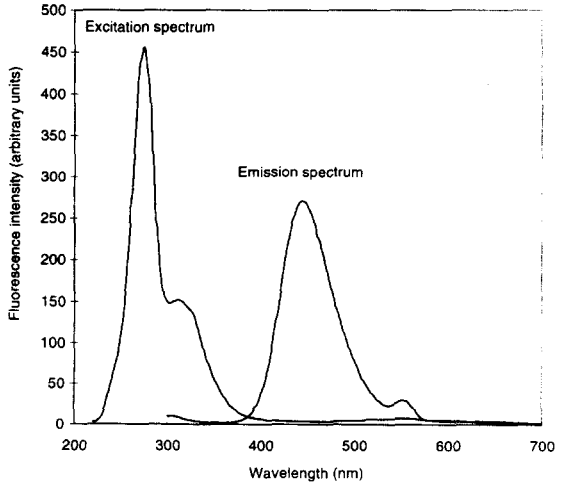

Fig. 2. Excitation and emission spectra of enrofloxacin. Emission spectrum was obtained with prior excitation at 315 nm.

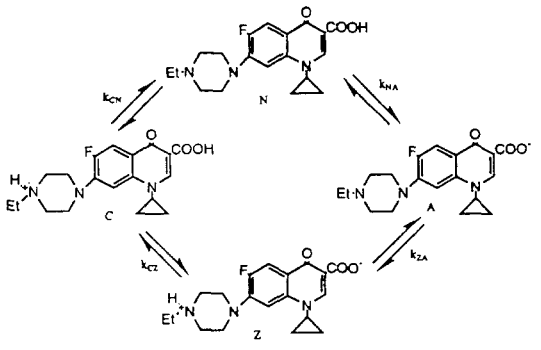

Fig. 3. Protolytic equilibria of enrofloxacin analogues.

3. Dissociation constants

Knowing the dissociation constants of a quinolone is important, since they help define the state of ionization of the quinolone at any given pH. This is important if the ability of a quinolone to be absorbed from the gastrointestinal tract or across other biological membranes is determined by the fraction of the quinolone in a particular ionic state.

Determinations of pK_a values were carried out according to conventional conductometric techniques [6]. On the day of the experiment, 0.55 mM enrofloxacin aqueous solution was titrated with 50 mM sodium hydroxide (to obtain the pK_{a1}) and with 50 mM hydrochloride acid (for the pK_{a2}). The analyses were performed in triplicate and the following values were obtained: $pK_{a1} = 5.94 \pm 0.09$ and $pK_{a2} = 8.70 \pm 0.44$. These values corresponded to the carboxylic acid group in the 3-position and the basic piperazinyl group in the 7-position, respectively. It is well established that for any fluoroquinolone the pK_a corresponding to the carboxylic acid group is around 6.0 ± 0.3 and relatively independent of the substitution at the 7-position [7].

Enrofloxacin can exist in four possible forms: as an acidic cation (**C**), a neutral un-ionized species (**N**), an intermediate zwitterion (**Z**) and a basic ion (**A**), depending on the given pH. At a low pH, both the 7-piperazinyl group and 3-carboxyl group are protonated. At a high pH, neither is protonated.

Fig. 3 expresses the protolytic equilibria of enrofloxacin analogues. In this figure, k_{CN}, k_{CZ}, k_{NA} and k_{ZA} represent the microdissociation constants, which are related to the macroscopic constants K_1 and K_2 by the equations:

$$K_1 = k_{CN} + k_{CZ} \tag{1}$$

$$1/K_2 = 1/k_{NA} + 1/k_{ZA} \tag{2}$$

Microdissociation constants were determined by absorbance changes caused by variations in pH of solutions of enrofloxacin in buffers of various pH values from 1.0 to 10.0 at 25°C. Water solutions were prepared from a two-solution buffer system (solution A: 0.2 M anhydrous boric acid and 0.05 M citric acid monohydrate; solution B: 0.1 M trisodium phosphate dodecahydrate), as is indicated elsewhere [8]. The absorbance at 276 nm measured at each pH studied allowed to the fraction of total carboxylic group dissociated, α, to be obtained with the equation:

$$\alpha(pH) = \frac{A_{COOH} - A_{pH}}{A_{COOH} - A_{COO^-}} \tag{3}$$

In the above equation, A_{COO^-} and A_{COOH} represent the values of absorbance at the extreme pH's (10 and 2, respectively) and A_{pH} the absorbance at a defined pH. The fraction α at any pH is related to the microdissociation constant k_{CN} by the following equation [9]:

$$(1 - \alpha_{pH}) = \frac{k_{CN}[H^+] + K_1K_2}{[H^+] + K_1[H^+] + K_1K_2} \tag{4}$$

After calculating k_{CN}, the other microconstants can be easily obtained from Eq. (1) and Eq. (2).

At each pH, the fractions of cationic (**C**), anionic (**A**) and neutral (**N'**) (uncharged (**N**)) and zwitterionic (**Z**) species can be calculated from the following equations [10]:

$$[\mathbf{C}] = \frac{[H^+]^2}{[H^+]^2 + K_1[H^+] + K_1K_2} \tag{5}$$

$$[\mathbf{N'}] = \frac{K_1[H^+]}{[H^+]^2 + K_1[H^+] + K_1K_2} \tag{6}$$

Table 2
Values of pK's associated with macro- and microconstants and fractions of the species of enrofloxacin present at isoelectric pH (pI)

pK_{a1}	5.94
pK_{a2}	8.70
$pI = (pK_{a1} + pK_{a2})/2$	7.32
pK_{CN}	7.11
pK_{CZ}	5.97
pK_{NA}	7.52
pK_{ZA}	8.67
[C]	0.0385
[A]	0.0385
[N']	0.9231
[N]	0.0620
[Z]	0.8611
[Z]/[N]	13.89

$$[\mathbf{A}] = \frac{K_1 K_2}{[H^+]^2 + K_1[H^+] + K_1 K_2} \quad (7)$$

The proportion of neutral species can be obtained by:

$$[\mathbf{Z}] = \frac{k_{CZ}[\mathbf{C}]}{[H^+]} \quad (8)$$

$$[\mathbf{N}] = \frac{k_{CN}[\mathbf{C}]}{[H^+]} \quad (9)$$

Table 2 summarizes the values of macro- and microdissociation constants, as well as the fractions of the different species of enrofloxacin; and

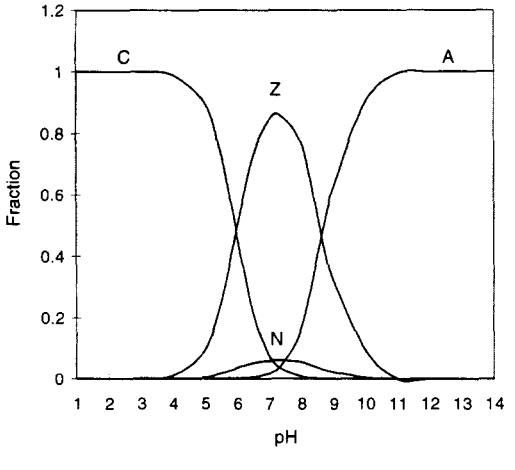

Fig. 4. Distribution of the fraction of the four species: the cation (**C**), the neutral (**N**), the zwitterion (**Z**) and the anion (**A**) of enrofloxacin as a function of pH.

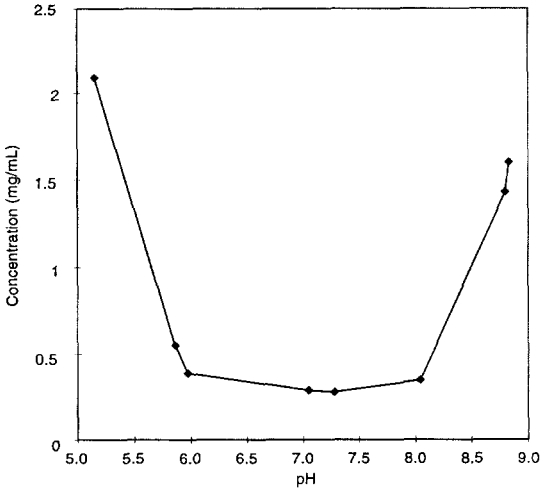

Fig. 5. Solubility/pH profile of enrofloxacin. The dots are the experimental values.

Fig. 4 shows how the fractions of the four possible forms can vary as a function of pH. In solution, cationic and anionic species approach the total concentration of enrofloxacin at high and low pH values, respectively, and neutral species reaches a maximum in the isoelectric pH. As the dissociation constant for the carboxylic acid is greater than for the amine, the neutral form is mainly the zwitterion. Uncharged neutral species are in extremely low proportion.

4. Solubility

Solubility studies were performed with the help of a rotating sample holder at 25°C. Solubility was determined over the pH range 2.05–8.83. Excess drug was added to the different buffer solutions, which were maintained at 0.1 ionic strength with NaCl. The solutions were placed in amber vials for light protection and agitated for 2 days. At the end of this period, the sample were withdrawn and filtered with 0.45 mm nitrocellulose membranes (Lida, USA). The pH's of filtered solutions were measured and their concentration assayed by UV-spectrophotometry. All solubility measurements were performed in triplicate.

Fig. 5 shows the pH–solubility profile of enrofloxacin which exhibits a zone of low solubility

in the neighbourhood of the isoelectric point. Maximal solubility was achieved at pH 5.02 (10.42 ± 0.96 mg/ml ≈ 28.98 mM). The amount of enrofloxacin solubilized can be increased using a more concentrated acetate buffer. In this way a 1.178 M acetate buffer is able to dissolve more than 100 mg ml^{-1} of enrofloxacin.

5. Partition coefficients

Partition coefficients at different pH's were determined by the flask-shaking method. Octanol and aqueous buffers were mutually pre-equilibrated before use. Enrofloxacin was dissolved directly in the organic-saturated buffer at 16 mg ml^{-1} concentration. The final concentration was checked and corrected if necessary, its absorbance (A) being determined at the wavelength of maximal absorption. A volume of this solution was thoroughly mixed with the same volume of buffer-saturated organic phase, the mixture was stirred for 120 min at room temperature and set aside for 30 min to facilitate phase equilibrium; the phases were then separated by centrifugating (1000 rpm, 10 min). An aliquot was accurately removed from the aqueous phase and its absorbance measured (A'). The apparent partition coefficient (P') was obtained from $P' = (A - A')/A'$.

Fig. 6 shows the apparent partition coefficients determined over the pH range between 2 and 10.

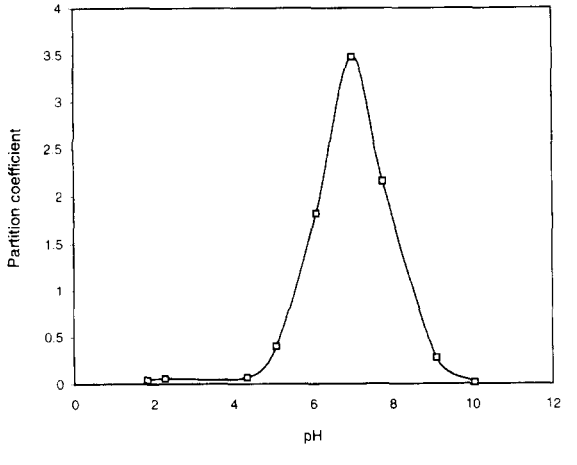

Fig. 6. Apparent partition coefficient of enrofloxacin at several pH.

They are the average of six parallel measurements. This curve has a peaked parabolic shape, typical of zwitterionic compounds [11].

The amount of drug transferred from the aqueous to the organic phase was maximal at pH 7.00 ($P' = 3.48 \pm 0.04$). Below and above this pH, partition coefficients decrease, which indicates that drug polarity is higher and, therefore, a minor transference. However, as Takács-Novák et al. reported [11], only in zwitterionic compounds does the true partition coefficient closely represent intrinsic lipophilicity. For such types of ampholytes the following equation allows the true partition coefficient (P) to be calculated from the apparent coefficient partition and the microprotonation constants:

$$\log P = \log P' + \log\left(1 + \frac{1}{k_{NA}[H^+]} + \frac{k_{CN}}{k_{CZ}} + k_{CN}[H^+]\right) \quad (10)$$

At pH 7.0, a $\log P = 4.70$ was calculated, a value that is more than one order of magnitude greater than the $\log P'$, since the concentration of neutral species is low in comparison with that existing in the aqueous phase.

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