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ORIGINAL ARTICLE

Enrofloxacin inclusion complexes with cyclodextrins

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Abstract Inclusion complexes using α-, β-, γ-, and hydroxypropyl-β-CD (HP-β-CD) were produced with the antibiotic enrofloxacin, with the aim of increasing its solubility by complexation. Phase solubility diagrams were obtained, to confirm the formation of inclusion complexes, and to determine the solubility enhancement and stability constant of each complex. Enrofloxacin inclusion in β-CD showed the highest value of the complex stability constant (35.56 mmol L^{-1}), but the greatest increase in solubility was obtained using HP-β-CD reaching a 1258% increase over enrofloxacin solubility in the absence of CD. The order of highest enrofloxacin solubility achieved was: HP-β-CD > α-CD > γ-CD > β-CD. In addition, formation of complexes was confirmed by differential scanning calorimetry and thermogravimetry, applied to the complexes obtained by the kneading technique. The influence of citric acid, alone or as an adjunct of β-CD, on the solubility of enrofloxacin was also determined. A solution of 15 mmol L^{-1} citric acid dissolved 10 g L^{-1} of enrofloxacin, but a gradual increase in β-CD concentration in the presence of citric acid did not increase the degree of solubilization of enrofloxacin.

Keywords Enrofloxacin · Cyclodextrin · Inclusion complex · Stability constant

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Introduction

The development of the fluoroquinolone class of antibiotics has been a major breakthrough in the treatment of bacterial infections. It has a wide spectrum of antibacterial activity against organisms that are resistant to many other antibacterial substances. Enrofloxacin (Fig. 1) is a fluoroquinolone antibiotic and has shown efficacy for veterinary use. The very poor aqueous solubility and wettability of enrofloxacin, gives rise to difficulties in the design of pharmaceutical formulations and leads to variable bioavailability [1, 2]. The problem of low solubility of many drugs has been overcame by complexation of the active principle with cyclodextrins (CDs) [3] and this work was developed aiming to study the application of this procedure to enrofloxacin, as to our knowledge, this has not been attempted before.

CDs are cyclic oligosaccharides composed of a variable number of α-1,4-D-glucopyranosyl residues, linked by α-1,4 bonds. The most common CDs have six, seven, or eight residues and are called α-, β-, or γ-CD, respectively. The CD molecules are externally hydrophilic and relatively hydrophobic inside their ring cavity. In liquid or occasionally solid media, CDs are able to form inclusion complexes with many different types of appropriately sized, preferentially nonpolar molecules [5–7]. They have received considerable attention in pharmaceutical application because they improve water solubility, chemical stability, and bioavailability of various drugs through the formation of inclusion complexes [8]. CDs can be applied to reduce the negative effects of bitter or irritant tasting and bad smelling drugs. Additionally, their potential for increasing the stability of included molecules against hydrolysis, oxidation, photodecomposition and dehydration warranted the encapsulation studies. Natural CDs, in

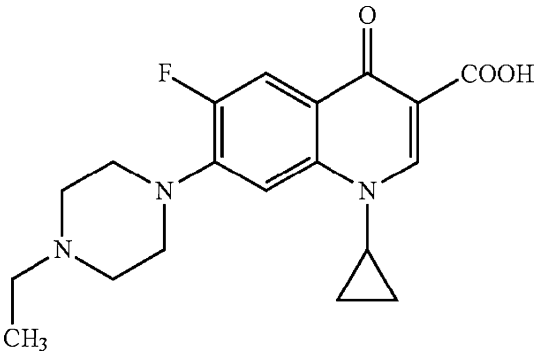

Fig. 1 Structural formula of enrofloxacin [4]

particular β-CD, are of limited aqueous solubility. Substitution of any of the hydrogen bond-forming hydroxyl groups, even by lipophilic functions, results in dramatic improvement in their aqueous solubility. Hydroxypropyl-β-CD (HP-β-CD) is a common and typical kind of β-CD derivative. It maintains the inclusion complex formation property of β-CD and improves its solubility at the same time. Derivatization, such as that in HP-β-CD, increases aqueous solubility by imparting greater flexibility to the external hydroxyl groups, which also have the potential to increase hydrogen bond formation between the guest molecule and the derivatized CD and, consequently, may increase the stability of the inclusion complex. With a relatively low price, HP-β-CD has a broad prospect in practical application [6, 7, 9, 10].

The most widely used approach to study inclusion complexation is the phase solubility method described by Higuchi and Connors [11] which examines the effect of a solubilizer (CD or ligand) on the drug being solubilized, that is, the substrate [8]. Given the low solubility of enrofloxacin in water, the viability of producing an inclusion complex of this substance in CDs was studied in this work. The influence of citric acid, used alone or as an adjunct of β-CD, on the solubility of enrofloxacin was also determined. Previous studies have already reported a synergistic effect of organic acids and CDs on the solubility of drugs, especially with citric acid. This type of solubility is generally attributed to a change in the solute–solvent interaction, such as ionization of guest molecules [7].

Materials and methods

Materials

The CDs used in this work were α-CD from Fluka Chemie AG (Sweden), β-CD and HP-β-CD from Sigma Chemical Co. (EUA), and γ-CD from Wacker Consortium (Germany). Filtering membranes (0.22 μm) were from Millipore. Enrofloxacin was a gift from Formil Química Ltda (Brazil). Other analytical grade reagents and distilled water were used throughout this work.

Enrofloxacin absorption spectra and solubility

A low-concentration enrofloxacin aqueous solution (0.02 g L^{-1}) was initially prepared and its absorption spectra (190–1100 nm), before and after membrane filtration (0.22 μm) at 30 °C were compared, in order to verify whether all the material had been dissolved and to determine the maximum absorption wavelength (λ_{max}).

Enrofloxacin solutions from 0 to 0.1 g L^{-1} were prepared with distilled water and the absorbance was measured at λ_{max}. The enrofloxacin spectrophotometric analysis results remained linear up to the concentration of 0.025 g L^{-1}. A straight line was fitted to the data, yielding enrofloxacin specific molar absorbance (ε) as the slope. A Shimadzu UV-1601PC spectrophotometer was used for the absorption measurements.

The water solubility limit for enrofloxacin was determined at 30 °C: A suspension of 10 g L^{-1} of enrofloxacin was prepared with distilled water and agitated for 5 days at 150 rpm and 30 °C. After this period, the solution was filtered with a 0.22 μm membrane and the filtrate was analyzed at λ_{max}, yielding the enrofloxacin solubility.

Phase solubility tests

Phase solubility measurements were carried out according to the method of Higuchi and Connors [11], using solubility diagrams to show whether inclusion complexes were formed and the guest/host molar ratio of the formed complex. CDs solutions were prepared with initial concentrations close to their solubility limits (g L^{-1}): α-CD, 120.80; β-CD, 18.00; γ-CD, 207.50 and HP-β-CD, 600.00. Each one of these solutions was diluted to different concentrations and enrofloxacin was added (0.1000 g), forming 10 mL volumes. After homogenization, solutions were kept under agitation (150 rpm) at 30 °C for 5 days. The solutions were then filtered and the filtrate was diluted and analyzed by spectrophotometry. Absorbance measurements were used to calculate the solubility data plotted in Fig. 2. For each CD used, a straight line was fit to the data and Equ. 1 was applied for calculating the complex stability constant (K), while Equ. 2 gave the enrofloxacin solubility enhancement ratio upon CD addition (n). *Slope* is the angular coefficient of the fitted straight line; S_0 is the intercept; and S_{max} is the maximum enrofloxacin concentration in the filtrate after CD addition.

$$K = \frac{\text{Slope}}{S_0(1 - \text{Slope})} \quad (1)$$

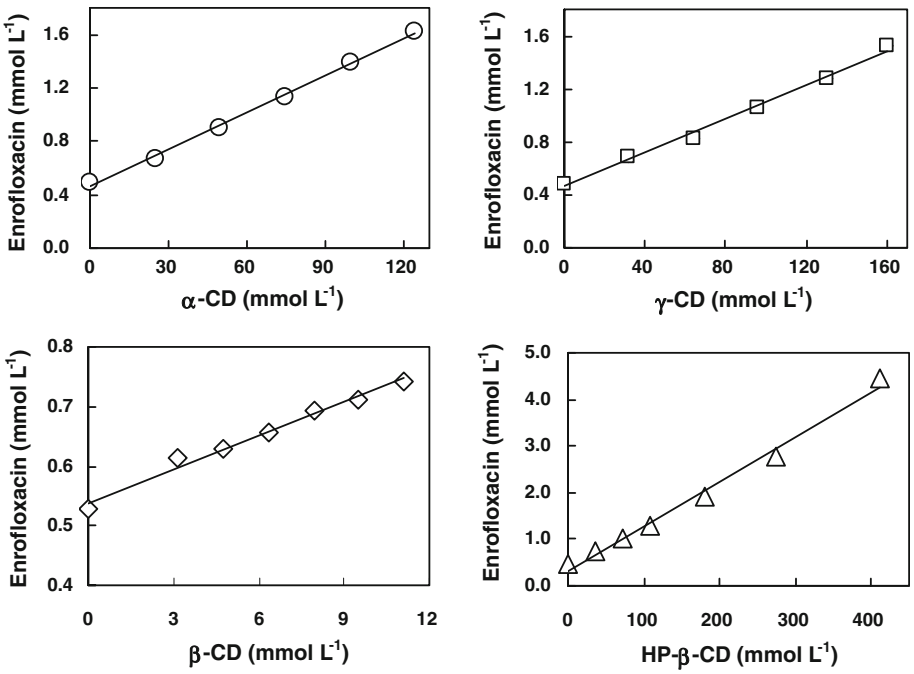

Fig. 2 Enrofloxacin solubility in water at 30 °C, as a function of CD concentration

$$n = \frac{S_{max}}{S_0} \quad (2)$$

Differential scanning calorimetry (DSC) and Thermogravimetric analysis (TGA) analyses

Thermal analyses were used for confirming CDs:enrofloxacin complexation. TGA and DSC curves were obtained under dynamic nitrogen atmosphere (30 mL min^{-1}), at a heating rate of 10 °C min^{-1} (20–500 °C), using a NETZCH STA 409 PC/PG equipment.

Samples for thermal analyses were prepared by the kneading complexation technique: enrofloxacin complex with each CD (1:1 molar ratio) was prepared by kneading 1 g of CD with 1 mL of water, at ambient temperature, up to homogenization (~5 min). Then, enrofloxacin was added to the paste and kneading continued for a further period of 20 min. The resulting paste was dried in a desiccator at ambient temperature (25 °C) for 48 h.

Enrofloxacin solubility in the presence of citric acid and β-CD

The objective of this assay was to determine the influence of citric acid on the solubilization of enrofloxacin, since it changes the pH and the solubility of the drug. Citric acid was also used as a potential auxiliary agent in the complexation of enrofloxacin with β-CD. The determination of the influence of citric acid alone on the solubility allowed to discriminate its influence on the complex formation and to quantify the effect of adding β-CD after the addition of citric acid.

(a) For the tests in which citric acid was used for enhancement of enrofloxacin solubility, the same procedure as with the phase solubility tests was carried out, in the first case, exchanging the CDs for citric acid at the concentrations of 1, 2, 5, 10, 15, 20, and 50 mmol L^{-1}.
(b) In the second case, in order to verify if citric acid increases the solubility of enrofloxacin in the presence of β-CD, 5 mmol L^{-1} of acid were used, varying the concentration of β-CD from 0 to 18.00 g L^{-1} and adding 0.1000 g of enrofloxacin, with a final volume of 10 mL.

Results and discussion

Enrofloxacin absorption spectra and solubility

The absorption spectra for the filtered and not filtered 0.02 g L^{-1} enrofloxacin solutions were coincident, showing that all enrofloxacin was solubilized. The wavelength of maximum absorbance and the specific molar absorbance were determined ($\lambda_{max} = 272$ nm and $\varepsilon = 35.02$ L $mmol^{-1}$ cm^{-1}).

Enrofloxacin solubility, determined in water at 30 °C, was 0.173 ± 0.006 g L^{-1}. Seedher and Agarwal [2] reported a solubility of 0.146 g L^{-1} for enrofloxacin at 25 °C.

Phase solubility tests

Phase solubility diagrams corresponding to the enrofloxacin:CD systems (CD = α, β, γ, or HP-β-CD) are presented in Fig. 2. The linear increase in enrofloxacin solubility with increasing CD concentration is classified as type A_L according to Higuchi and Connors [11] and indicates the formation of a 1:1 stoichiometry of complexation. For a more definitive confirmation of molar complexation stoichiometry Job's plots are required [12, 13]. A straight line was fitted to the data and the equilibrium constants K and n, obtained by Equs. 2 and 3, are listed in Table 1.

The values of S_0 do not represent the intrinsic solubility of enrofloxacin in distilled water, but the intercept of the phase solubility diagrams and this explains why the value may be different for each CD tested. Loftsson et al. [14], indicate that S_0 coincide with the intrinsic solubility only for drugs with solubility greater than about 1 mol L^{-1}. Table 1 shows that this is not the case for enrofloxacin.

The greatest increase in enrofloxacin solubility was obtained using HP-β-CD ($n = 13.6$), representing 1258% increase over the intrinsic enrofloxacin solubility in the absence of CD. The order of highest enrofloxacin solubility achieved was: HP-β-CD > α-CD > γ-CD > β-CD. In spite of being the least soluble CD, β-CD formed the inclusion complex (enrofloxacin: β-CD) with the highest stability constant ($K = 35.56$ mmol L^{-1}). Although the increase in solubility (n) observed with the other CDs was greater than that of β-CD, this increase should be balanced by the fact that the quantities used of these CDs were much higher than that of β-CD (7–33 times), since they are more soluble. The most concentrated enrofloxacin solution was obtained with HP-β-CD (4.46 mmol L^{-1}), while with β-CD the concentration was 0.7420 mmol L^{-1}. This result is consistent with the fact that HP-β-CD was the most soluble CD used in this work.

The values obtained for K indicate a relatively low stability of the complexes enrofloxacin:CD. Melo et al. [15] also obtained low values of K: 14.9 ± 2.3 and 10.8 ± 1.2 mol L^{-1} at 20 and 25 °C, respectively, for the complexation of nitrofurazone with HP-β-CD, the former being an antimicrobial compound. According to these authors, the reason for this low affinity may be due to the size and geometry of the CD cavity, the relative solubility, and the conformational energy of the drug. Interestingly, the presence of hydroxypropyl side groups in HP-β-CD

Table 1 Phase solubility diagram parameters (from Fig. 2), complex stability constant (K) and enrofloxacin solubility enhancement ratio upon CD addition (n)

CD	α-CD	β-CD	γ-CD	HP-β-CD
Slope	0.0093	0.0188	0.0064	0.0096
S_0 (mmol L^{-1})	0.4574	0.5388	0.4614	0.3281
S_{max} (mmol L^{-1})	1.6245	0.7420	1.5301	4.4551
R^2	0.9976	0.9861	0.9923	0.9904
K (L mol^{-1})	20.52	35.56	13.96	29.54
n	3.6	1.4	3.3	13.6
Solubility enhancement (%)	255	38	232	1258

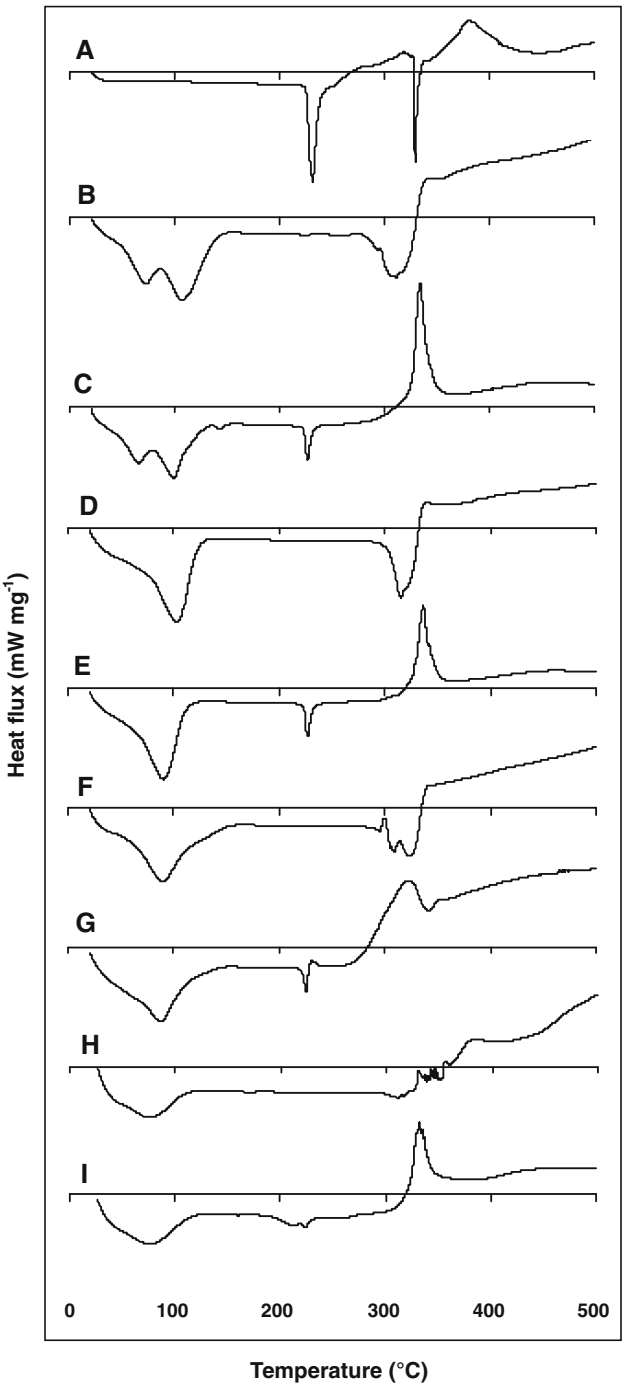

Fig. 3 DSC thermogram for enrofloxacin (*A*), α-CD (*B*), β-CD (*D*), γ-CD (*F*), HP-β-CD (*H*), and the products obtained by the complex preparation using the kneading technique (1:1 molar ratio), with enrofloxacin and: α-CD (*C*), β-CD (*E*), γ-CD (*G*), and HP-β-CD (*I*)

reduces the affinity of the CD for enrofloxacin, since the complexation constant decreased from 35.56 mol L^{-1} for β-CD to 29.54 mol L^{-1} in the case of HP-β-CD.

DSC and TGA analyses

Thermoanalytical methods are suitable to ascertain whether a particular product is a true complex. They determine if the guest substance undergoes some change before the thermal degradation of CD (250–300 °C). This change may be evaporation, decomposition, oxidation, melting, or polymorphic transition [16].

Figure 3 shows the DSC curves obtained for each CD, along with the curves for pure enrofloxacin and for the complexes enrofloxacin:CD (1:1 molar ratio) obtained using the kneading technique. The thermogram of enrofloxacin (curve A) presents two endothermic peaks: the first at 231 °C corresponds to the melting point and the second at 329 °C to the flash point. The thermograms of the CDs (curves B, D, F, and H) present an endothermic peak between 80 and 100 °C, associated with loss of moisture and another one between 315 and 330 °C, caused by thermal degradation of the CD. The thermograms of the enrofloxacin:CD complexes (curves C, E, G, and I), show a reduction of the peak corresponding to the enrofloxacin melting point. This reduction is partially due to the larger mass of CD that was used in relation to the amount of enrofloxacin in order to obtain a 1:1 molar ratio. However, as the reduction of peak area was considerable, especially for enrofloxacin:γ-CD and enrofloxacin:HP-β-CD complexes, the formation of complexes was confirmed. Moreover, the peaks above 300 °C, ascribed to the flash point of enrofloxacin and thermal degradation of CDs, changed from endothermic to exothermic, indicating that a different species had been formed (probably the inclusion complex).

In the TGA curves shown in Fig. 4, there is a small weight loss at 100 °C due to water evaporation and a gradual weight loss after 300 °C due to thermal degradation of enrofloxacin and CDs. The homogeneous displacement (without the presence of steps along the curve) observed for the enrofloxacin:CDs mixtures in relation to the curves of pure substances, is another indication of the formation of complexes.

Enrofloxacin solubility in the presence of citric acid and β-CD

Figures 5, 6 show a strong influence of the concentration of citric acid used alone, and associated solution pH, on the enrofloxacin solubility. The solubility test using 15 mmol L^{-1} of citric acid showed that 10 g L^{-1} of enrofloxacin (27.82 mmol L^{-1}) were completely solubilized. The solution pH was about 5.0. The observed increase in solubility, particularly in terms of pH, confirmed the results of Lizondo et al. [17], who obtained a maximum enrofloxacin solubility of 28.98 mmol L^{-1} at pH 5.02.

Citric acid alone at a concentration of 5 mmol L^{-1} increased the solubility of enrofloxacin to 10 mmol L^{-1}

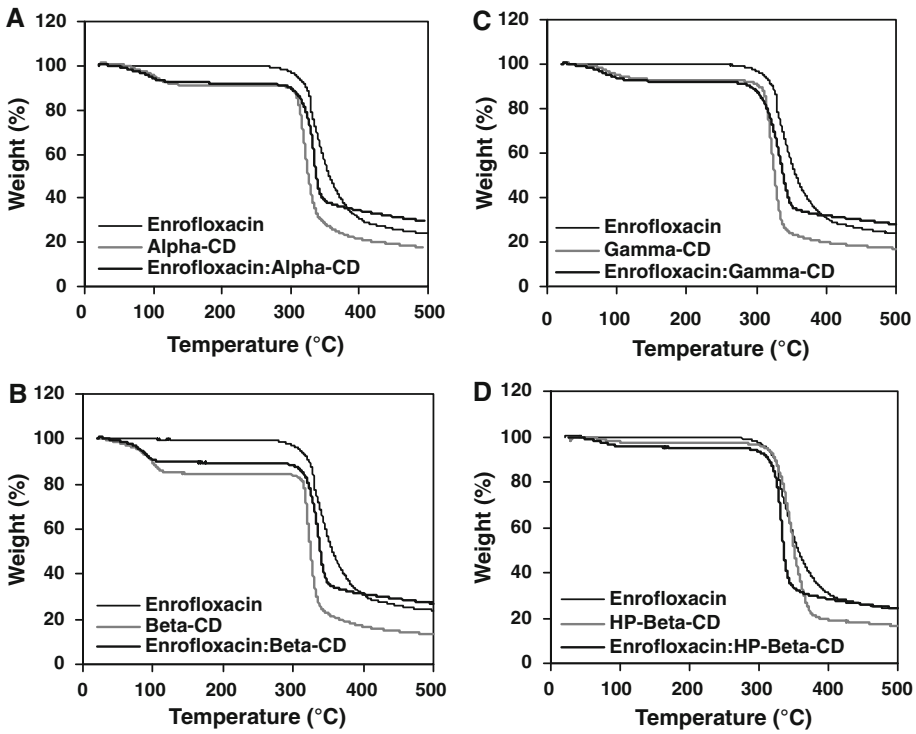

Fig. 4 Thermogravimetric curves of enrofloxacin, CDs (**A** = α-CD, **B** = β-CD, **C** = γ-CD, and **D** = HP-β-CD), and products obtained by the complex preparation using the kneading technique with enrofloxacin and each one of the CDs (1:1 molar ratio)

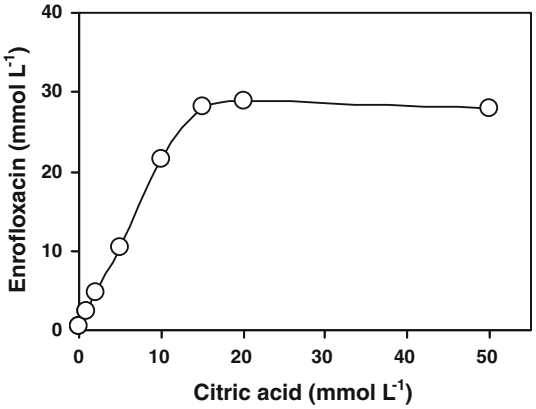

Fig. 5 Enrofloxacin solubility as a function of citric acid concentration

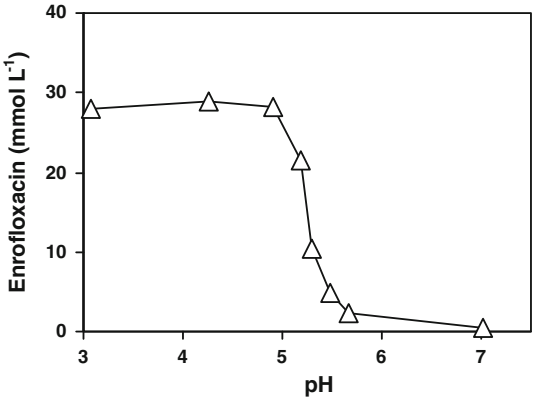

Fig. 6 Enrofloxacin solubility as a function of pH

(3.7 g L^{-1}). However, in the experiments with addition of citric acid and β-CD, the gradual addition of β-CD to the 5 mmol L^{-1} citric acid solution did not result in any increased solubility under the conditions of this test. The pH of the solutions remained around 5.3 after the addition of β-CD.

Conclusion

Using α-, β-, γ-, and HP-β-CD, enhanced solubility of enrofloxacin in water was achieved, with the formation of complexes of molar ratio 1:1, according to phase solubility studies. DSC and TGA analyses confirmed the formation of these complexes. Among the CDs tested, inclusion of enrofloxacin in β-CD yielded the highest complex stability constant ($K = 35.56$ L mol^{-1}). However, the greatest solubility of enrofloxacin, 1.60 g L^{-1} (4.46 mmol L^{-1}), was obtained with HP-β-CD, giving $n = 13.6$ and $K = 29.54$ L mol^{-1}. The solubility test using 15 mmol L^{-1} of citric acid showed that 10 g L^{-1} of enrofloxacin (27.82 mmol L^{-1}) was completely solubilized. The solution pH was about 5.0. An increase in β-CD concentration in the presence of 5 mmol L^{-1} citric acid did not increase the degree of solubilization of enrofloxacin.

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