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Enzymatic hydrolysis of diloxanide furoate in the presence of β-cyclodextrin and its methylated derivatives

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Abstract

In this study, we investigated the susceptibility to enzymatic and alkaline hydrolysis of diloxanide furoate (DF) and its cyclodextrin inclusion complexes, in aqueous solution. The cyclodextrins (CDs) utilized were β-cyclodextrin (β-CD), (2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) and (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD). All cyclodextrins studied provided a stabilizing effect to diloxanide furoate hydrolysis. In alkaline hydrolysis (pH 10.75), without the enzyme, β-CD and TM-β-CD provided similar effect on the stability of DF, with an inhibition factor in the order of 2.0. The DM-β-CD, on the other hand, provided more pronounced stabilization effect than the other two CDs, with an inhibition factor around of 8. The maximum activity of the enzyme occured around pH 7.0. In the presence of enzyme, all cyclodextrins produced similar effect, with a DF hydrolysis inhibition factor in the order of 10. However, the plot of rate of hydrolysis versus [CD] fit with a equation based in a model that considers the association of the enzyme with the CDs. Therefore, it is concluded that the stabilization of DF is not only due to its cyclodextrin complex but also due to enzyme inhibition by cyclodextrin complexation.

Keywords: Diloxanide furoate; Cyclodextrin; Enzymatic hydrolysis; Stability

1. Introduction

Diloxanide furoate (Furamide®), a chloroacetamide, 2-furoyl ester of diloxanide (2-furoyl 2,2-dichloro-4-hydroxy-*N*-methylacetanilide) is an antiparasitary agent which acts against cryptosporidiosis, an acute intestinal amoebiasis and one of the infections that afflicts patients with the human immunodeficiency virus (HIV). Therefore, it has been extensively investigated in recent years (Guarino et al., 1997; Ramratnam and Flanigan, 1997; Fricker and Crabb, 1998; Chaisson et al., 1998). This drug is orally administered and is poorly soluble in water. When administered to infected patients, it can be hydrolyzed by esterases of the gastrointestinal tract, thus diminishing its effectiveness. Studies on absorption and distribution of this amoebicide, in man and experimental animals, show that the diloxanide furoate is extensively hydrolyzed and the main urinary excretion product is diloxanide, less active than its furoyl ester, diloxanide furoate (Wilmshurst and Cliffe, 1964). This demonstrates that intestinal esterases can catalyze the hydrolysis of diloxanide furoate to dilox-

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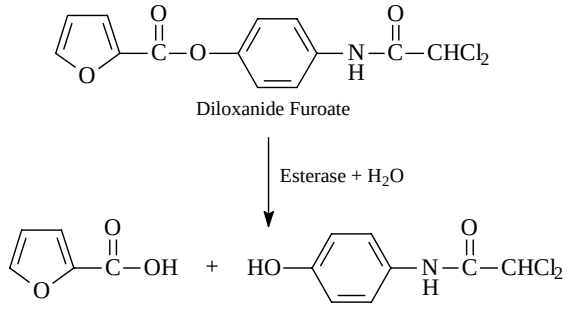


Fig. 1. Enzymatic hydrolysis of diloxanide furoate.

anide and furoic acid (Fig. 1). In this study, in order to improve the bioavailability of diloxanide furoate, we investigated the effect of β-cyclodextrin and its methylated derivatives on the alkaline and enzymatic hydrolysis of diloxanide furoate.

Cyclodextrins (CDs) can produce a catalytic (Berkel et al., 1998; Santos et al., 1999) or inhibitory (Bender, 1977) effect on enzymatic activity and/or act in the stabilization of organic compounds (Szejtli, 1984). In pharmaceutical area, cyclodextrins are known for their ability to form inclusion complexes with a variety of lipophilic drugs. This characteristic is due to their molecular structure and the spatial arrangement. Cyclodextrins possess a toroid overall shape with an apolar cavity, where the secondary hydroxyl groups, O(2)H and O(3)H, are located on one side of the molecule while the primary hydroxyl groups, O(6)H, are on the other side (Fig. 2). Cyclodextrins are used in the formulation of orally and topically applied drugs to improve the physical and chemical stability, bioavailability and to reduce the incidence of side effects (Szejtli, 1990; Stella and Rajewski, 1997; Veiga et al., 2000).

The anticancer drug doxorubicin and acetylsalicylic acid are unstable in aqueous solution; however, when complexed with γ- and β-cyclodextrin, respectively, they are more stable (Loftsson and Brewster, 1996). The effect of β-cyclodextrin and its derivatives on hydrolysis of aryl esters has been widely studied, as in the case, for example, of *p*-nitrophenyl α-methoxyphenylacetate (Park et al., 2000). Several studies have reported the interaction of cyclodextrins with peptidic drugs, protecting them against degradation by proteases; for example, the synthetic leutinizing hormone, [D-Trp^6,Des-Gly^{10}] LHRH Ethylamide, know as deslorelin, with hydroxypropyl β-cyclodextrin (Koushik et al., 2001); desmopressin with α, β e γ-hydroxypropyl cyclodextrins (Fredholt et al., 1999) and buserelin acetate with maltosyl-β-cyclodextrin (Matsubara et al., 1997). In these cases, the authors suggested that cyclodextrin complexation of amino acids of the lateral chains of the peptides is responsible for the stabilization of the peptide drugs undergoing catalyzed hydrolysis by protease, α-chymotrypsin. In case of buserelin acetate, the authors also argue a possible inhibition of the enzyme by cyclodextrin. A comparative study of β-CD, DMβ-CD and TMβ-CD complexes of flurbiprofen showed that methylated cyclodextrins are even more effective in improving the pharmaceutical properties of this drug than the unsubstituted β-cyclodextrin (Szejtli, 1988).

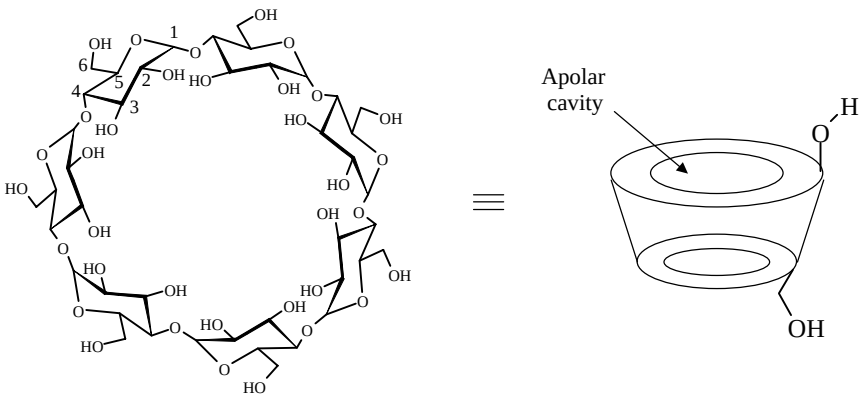


Fig. 2. Structure and schematic representation of β-cyclodextrin.

Studies carried out "in vivo" with diloxanide furoate, with and without β-cyclodextrin, by Blanco and coworkers (Castro Hermida et al., 2001) showed that diloxanide furoate is hydrolyzed in both cases, and that β-CD demonstrated a stabilizing and/or inhibitory effect.

In this work, a comparative study of the effect of three cyclodextrins, β-CD, DMβ-CD and TMβ-CD, on the stability of diloxanide furoate was investigated for both alkaline (pH 10.75) and enzymatic (pH 7.0) hydrolysis.

2. Materials and methods

2.1. Chemicals

Diloxanide furoate (DF) was supplied by Lab. Knoll S. A. Cyclodextrins, β-cyclodextrin (β-CD), (2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) and (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD), were kindly provided by Cerestar, USA. The hydrolytic enzyme, *Candida cilindracea* lipase (protease and α-amylase free) was purchased from Sigma Co. All chemicals used were of analytical grade.

2.2. Apparatus

Ultraviolet spectral measurements were performed with a Shimadzu UV-spectrophotometer equipped with a thermostated cell compartment, using 1.0 cm quartz cuvettes. The pH measurements were carried out with a digital potentiometer Micronal B 374, equipped with an Ingold double electrode, previously calibrated with standard solutions, Carlo Erba.

2.3. Hydrolysis of diloxanide furoate (DF)—kinetic measurements

The hydrolysis of DF (6.65×10^{-5} M) was carried out in an aqueous solution at appropriate pH and at 37 °C. For all reactions, an ionic strength of 0.5 M was maintained by adding a calculated amount of 2.5 M KCl. The reactions were performed in 3.0 ml aliquot portions of solutions in a thermostated quartz cuvette and the reactions were initiated by adding 20 μl of a 10^{-2} M solution of DF in acetonitrile to give a final concentration of 6.65×10^{-5} M. The progress of the reaction was followed by UV spectrophotometry ($\lambda = 259$ nm). The rate of hydrolysis of the ester was followed by monitoring the decrease in absorbance during at least three half-lives. The kinetic data, absorbance versus time, were stored directly on a microcomputer using a "Microquimica 12 bit A/D" interface board, and pseudo-first-order rate constants, k_{obs}, were estimated from linear plots of $\ln(A_\infty - A_t)$ using an iterative least squares program; correlation coefficients (r) were >0.999 for all kinetic runs. The dissociation constants, k_{diss}, were determined by linear estimation performed by *Origin* program package (MicroCal Origin version 5.0), the correlation coefficient (r) was utilized as statistical parameter. The experiments were carried out in triplicate. The pH effect, in pH range 5.5–10.0, maintained with the following buffer solutions: acetate (pH 5.5); phosphate (pH 6.0–7.5); borate (pH 8.0–9.5); bicarbonate (pH 9.5–10.0), on the enzymatic hydrolysis of DF in absence and in the presence of β-cyclodextrin (8.66×10^{-3} M) were studied. The enzyme concentration effect, in the absence of cyclodextrin, was effectuated in the range $(0.4–10.0) \times 10^{-3}$ g l^{-1}. The enzymatic hydrolysis with CDs was performed at pH 7.0 in the presence of 5.0×10^{-3} g l^{-1} of lipase. The alkaline hydrolysis was carried out at pH 10.75, in the absence of enzyme.

3. Results and discussion

3.1. Enzymatic hydrolysis of diloxanide furoate

3.1.1. pH effect

Studies of pH effect on enzymatic hydrolysis of DF were carried out in pH range 5.5–10.0, with and without β-CD at the concentration where saturation occurs. The pH-rate profile for the enzymatic hydrolysis of DF (Fig. 3) shows a strong pH-dependence, with a maximum at pH 7.0. At pH higher than 9.0, we could observe that the enzyme was considerably less active and basic catalysis was predominant. In the presence of β-CD (8.66×10^{-3} M), for maximum values of enzymatic activity, pH 7.0, the hydrolysis was stabilised by a factor of around 10.

3.1.2. Enzyme concentration effect

In the enzyme concentration range studied (0.4–10.0×10^{-3} g l^{-1}), the enzymatic hydrolysis of DF

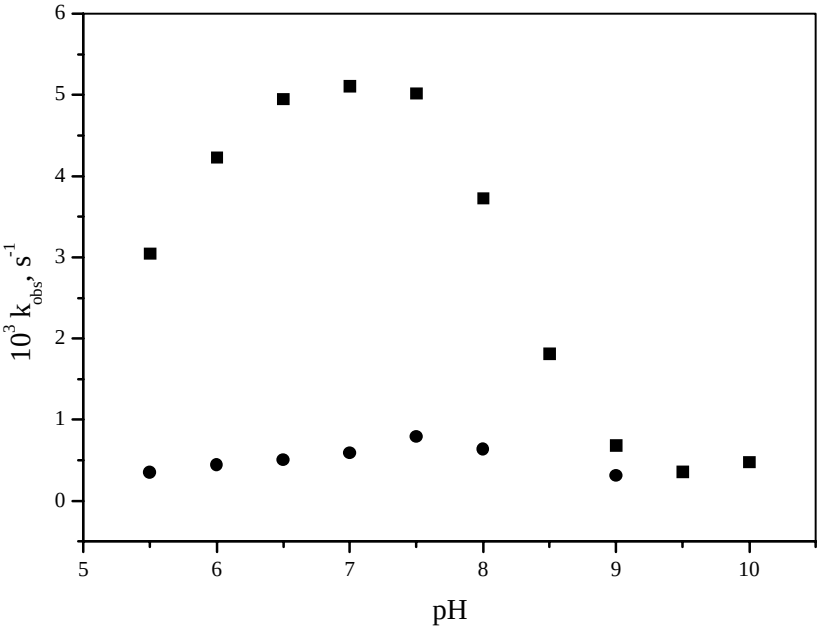


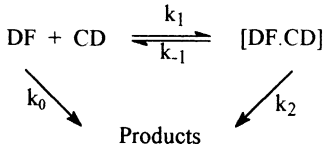
Fig. 3. pH-dependence of k_{obs} for enzymatic hydrolysis reaction of DF in aqueous solution, without cyclodextrin (■) and with β-CD (8.66×10^{-3} M) (●) at 37 °C and ionic strength 0.5 M.

without cyclodextrin shows a first-order kinetic, type Michaelis-Menten, Fig. 4. This was confirmed by measuring k_{obs} as a function of the enzyme concentration (C_E) and plotting the data according to the Lineweaver-Burk equation, obtaining a correlation coefficient (r) of 0.998. In the absence of enzyme, at pH 7.0, hydrolysis of DF occurred very slowly. The enzyme concentration used in the DF hydrolysis reactions, in the presence of cyclodextrins, subsequently presented, was 5.0×10^{-3} g l^{-1}.

3.2. Alkaline hydrolysis (pH 10.75) of diloxanide furoate in the presence of cyclodextrins

Three cyclodextrins, β-CD, DM-β-CD and TM-β-CD, showed saturation kinetics (Fig. 5) and the observed first-order rate constants (k_{obs}) asymptotically approached a minimum value with increasing cyclodextrin concentration. The alkaline hydrolysis of DF was considerably slower in the presence of DM-β-CD. For CD concentration of 8.66×10^{-3}, β-CD and TM-β-CD has a similar effect on the stability of DF, with an inhibition factor in the order of 2.0. The DM-β-CD has a much more pronounced stabilization effect than the other two CDs, with an inhibition factor around 8.

This saturation feature is characteristic of enzyme-catalyzed reactions, in which an excess of cyclodextrin should be present to ensure the conditions of a first order reaction. For the formation of a 1:1 complex, the following equilibrium applies:



Applying the steady state principle and for [CD] ≫ [DF],

$$K_{ass} = \frac{[DF \cdot CD]}{[DF] \cdot [CD]}$$

where, [CD] is the total cyclodextrin concentration in the solution and [DF·CD] is the DF-cyclodextrin 1:1

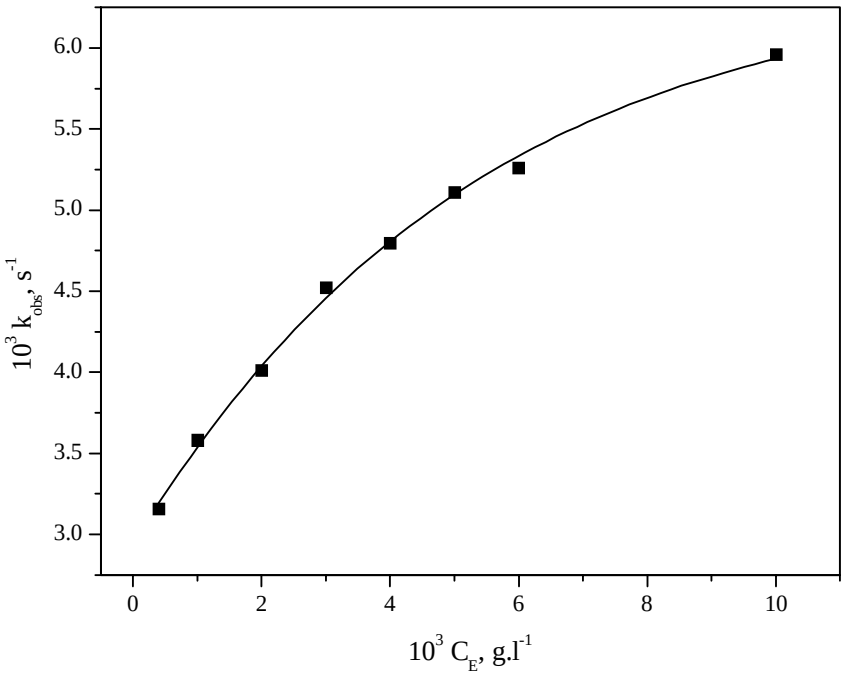


Fig. 4. k_{obs} vs. enzyme concentration for hydrolysis of DF at pH 7.0 and ionic strength 0.5 M.

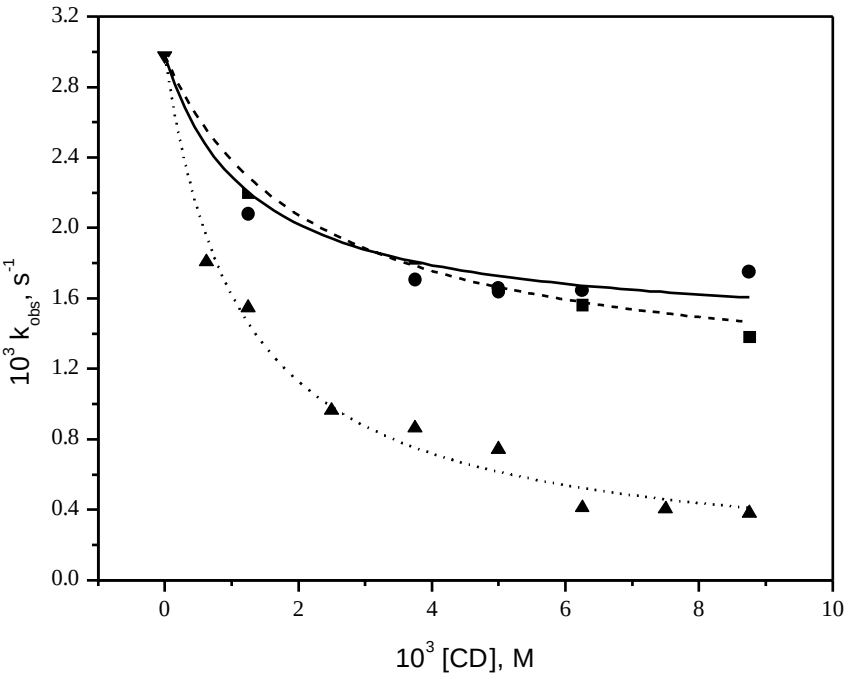


Fig. 5. Effect of β-CD (●), DM-β-CD (▲) and TM-β-CD (■) in DF alkaline hydrolysis reaction, at pH 10.75, 37 °C and ionic strength 0.5 M. For all CDs, $R^2 > 0.96$.

complex. The degree of stabilization of DF by CD complexation was dependent on both the hydrolysis rate of free DF within the solution (k_0) and within the complex (k_2), as follows:

$$k_{obs} = k_0 \cdot \chi_{DF}^{w} + k_2 \cdot \chi_{DF}^{CD} \quad (1)$$

where, the binding of DF to CD determines the contributions of k_0 and k_2 to the observed rate constants. Expressing the molar fractions of substrate (DF) in water and in CD in terms of $K_{diss,}$ the solution of Eq. (1) gives the following expression:

$$k_{obs} = k_0 + (k_2 - k_0)\frac{[CD]}{K_{diss(S)} + [CD]} \quad (2)$$

where, k_0 is the observed first-order constant for the hydrolysis of the free DF, k_2 is the rate constant for the hydrolysis in the presence of cyclodextrin, and K_{diss} is the dissociation constant of the complex [DF·CD]. The association constant (K_{ass}) is given by the relationship $K_{ass} = 1/K_{diss}$. The k_2 and K_{ass} values were determined by plotting the data according to the Lineweaver-Burk method ($1/(k_{obs} - k_0)$ versus 1/[CD]), Eq. (3).

$$\frac{1}{(k_{obs} - k_0)} = \frac{K_{diss(S)}}{(k_2 - k_0)}\frac{1}{[CD]} + \frac{1}{(k_2 - k_0)} \quad (3)$$

In alkaline hydrolysis, the results presented in Fig. 5 show that the association of DF with the CDs studied is related with the hydrophobicity and the steric hindrance close to the cyclodextrin cavity. The binding mechanism of the nonpolar guest-CD complexation is of hydrophobic nature. The DF-CD association constant values, calculated using a double reciprocal plot (Eq. (3)) are: 877, 517 and 461 M^{-1} for DM-β-CD, β-CD and TM-β-CD, respectively. A stronger association was observed for DM-β-CD than for β-CD and TM-β-CD. The DM-β-CD, which possesses one of the secondary hydroxyls, O(2)H, of each glucopyranose unit, and primary hydroxyls O(6)H, methylated, was more efficient in the improvement of the stability of the nonpolar diloxanide furoate. The methyl groups confer a hydrophobicity close to the extremity of the hydrophobic cavity of the cyclodextrin and greater flexibility to its structure. Another important factor to consider is the presence of intramolecular hydrogen bonds between an O(2)H hydroxyl group and O(3)H of the adjacent glucopyranose unit in β-CD (Endo et al., 1999). The low association constant value calculated for β-CD, compared to that of methylated CDs, is apparently due to these hydrogen bonds that make to β-CD less soluble and more rigid molecule and consequently, a reduced capacity to form the inclusion complex. The methylation of β-CD prevents the formation of hydrogen bonds and, consequently, the structure of the DM-β-CD becomes more flexible and hydrophobic than β-CD, which facilitate the formation of the inclusion complex. This yields a higher association constant. In case of the TM-β-CD, where all hydroxyl groups are methylated, the methyl groups located in the secondary face cause a steric hindrance and, consequently, a lesser drug-TM-β-CD association and a lesser stabilization effect on the DF, compared to the DM-β-CD. As the CD cavities are nonpolar and diloxanide furoate has a hydrophobic character, it is expected that the hydrophobic interaction provides the greatest contribution to the driving force in DF-CD inclusion complex formation. The importance of the hydrophobic interaction in CD complexation is also reported by Liu and Guo (2002).

3.3. Enzymatic hydrolysis of diloxanide furoate in the presence of cyclodextrins

In enzymatic hydrolysis of DF in the presence of the cyclodextrins, it was observed that the hydrolysis of DF was stabilized by the three CDs and yielded an effect typical of saturation kinetics (Fig. 6). A similar effect was observed with all cyclodextrins, β-CD, DM-β-CD and TM-β-CD. The stability factor, k_0/k_{obs}, for the three CDs was of the order of 10. This value is in agreement with those obtained in the studies on the effect of pH in the presence of β-CD at pH 7.0, at the concentration where saturation occurs (Fig. 3).

The experimental data do not adhere to Eq. (3), which suggests that the enzyme may be inhibited by CDs. This inhibition can occur with the complexation of amino acids of the lateral chains that participate in the active site of the enzyme, as reported by other researchers in studies involving the use of cyclodextrins with serine hydrolases such as α-chymotrypsin (Matsubara et al., 1997), with pancreatic amylase and with pullulanase (Bender, 1977).

Considering the association of both DF and the enzyme with CDs, as shown below:

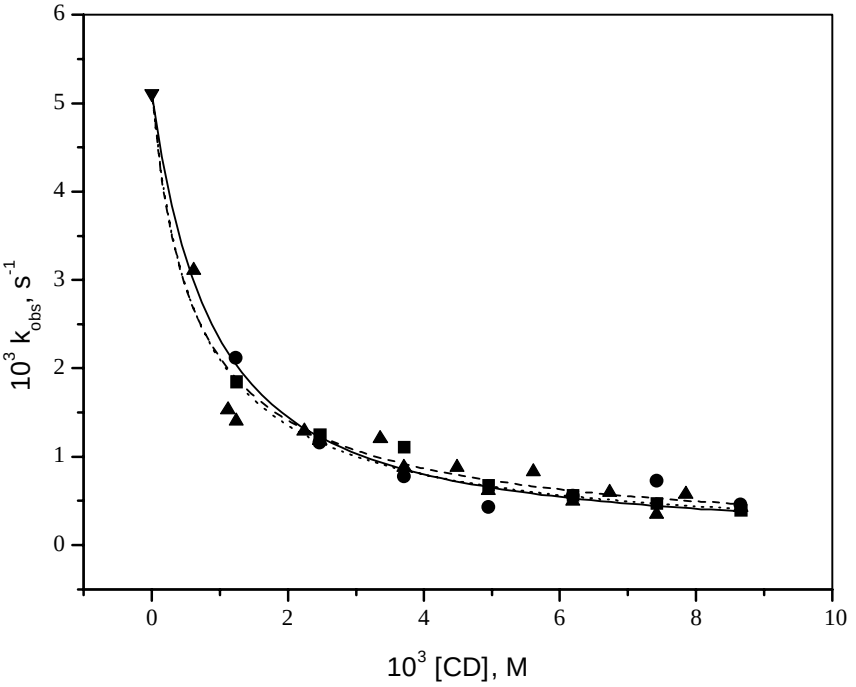
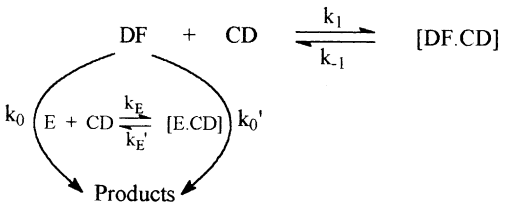


Fig. 6. Effect of β-CD (●), DM-β-CD (▲) and TM-β-CD (■) in DF enzymatic hydrolysis reaction, at pH 7.0, 37 °C and ionic strength 0.5 M. For all CDs, $R^2 > 0.97$.



the rate of the reaction can be expressed by Eq. (4):

$$k_{obs} = k_0 \cdot \chi_{free}^{DF} \cdot \chi_{free}^{E} + k_0' \cdot \chi_{free}^{DF} \cdot \chi_{comp}^{E} \quad (4)$$

where,

$$\chi_{free}^{DF} = 1 - \frac{[CD]}{K_{diss(DF)} + [CD]} \quad (5)$$

Substituting Eq. (5) in Eq. (4), we have:

$$\theta = \frac{k_{obs}}{\chi_{free}^{DF}} = k_0 + (k_0' - k_0)\frac{[CD]}{K_{diss(E)} + [CD]} \quad (6)$$

where, $k_{diss(E)}$ is the dissociation constant for the enzyme with CD and $k_{diss(DF)}$ is the dissociation constant for the substrate, diloxanide furoate, with CD, calculated as described previously, in item 3.2. Rearranging Equation 6 and applying a double reciprocal ($1/(\theta - k_0)$ versus 1/[CD]), we have Eq. (7), below:

$$\frac{1}{(\theta - k_0)} = \frac{K_{diss(E)}}{(k_0' - k_0)}\frac{1}{[CD]} + \frac{1}{(k_0' - k_0)} \quad (7)$$

According to the above model, theoretical curves fit with the experimental data (Fig. 6) and the association constants for the enzyme with the CDs, $K_{ass(E)} = 1/K_{diss(E)}$, were 794, 2421 and 2165 M^{-1} for β-CD, DM-β-CD and TM-β-CD, respectively. These results show that the enzyme inhibition, due to cyclodextrin complexation, contributes greater to the stabilization of diloxanide furoate. It is important to observe that the inhibition is partial, even at saturation, and the CD concentration necessary to inhibit 50% of hydrolysis of the DF is around 1.0×10^{-3} M, less than the saturation concentration of approximately 8.0×10^{-3} M. These differences in the behavior of the two systems, with and without CDs, can be due to the protective effect of the complexes against the esterases, as related by Wilmshurst (1963) and Blanco (2001) in "in vivo" studies.

4. Conclusion

The stabilization of diloxanide furoate is not only due to its cyclodextrin complexation but also due to enzyme inhibition by enzyme–cyclodextrin complexes. This shows the importance of the investigation in the presence of enzyme. The studied cyclodextrins, β-cyclodextrin and its methylated derivatives DM-β-CD and TM-β-CD, can improve the bioavailability of diloxanide furoate. In conclusion, it is possible to suggest that the CDs can stabilize DF through the formation of inclusion complexes as well as the inhibition of enzymes present in the gastrointestinal tract, improving the effect of this drug. These results also demonstrate that stability studies of drugs susceptible to being hydrolysed "in vivo" by enzymes present in animal tissue should be made taking into account enzyme–cyclodextrin interactions.

Acknowledgements

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