Cyclodextrins as Diethylstilbestrol Carrier System: Characterization of Diethylstilbestrol-Cyclodextrins Complexes

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Purpose. The *in vitro* formation of DES-cyclodextrins inclusion complexes was characterized using lipoxygenase as enzymatic system.
Methods. DES-cyclodextrins complexes were obtained in aqueous solution.
Results. The addition of cyclodextrins to the reaction medium had an inhibitory effect on DES oxidation by lipoxygenase due to the drug's complexation into the cyclodextrin cavity. This inhibitory effect depends on the complexation constant between DES and the cyclodextrins type used. In this case, β-, 2-hydroxypropyl-β- and γ-cyclodextrins have similar complexation constants and therefore produce the same inhibitory effect. Moreover, depending on the type of cyclodextrins used, the solubility of DES can be enhanced up to 956 times, while the lipoxygenase activity remains constant.
Conclusions. These results suggest that the system described may be used as a controlled-release delivery system for DES, since it may diminish the local and systemic adverse side effects caused by high concentrations of the drug.

KEY WORDS: DES; cyclodextrins; lipoxygenase; hydroperoxidase; H_2O_2; xenobiotics.

INTRODUCTION

Diethylstilbestrol (DES) is an inexpensive synthetic estrogen designed and used for medical purposes. Its first medical use was as a substitute for endogenous estrogens and as a hormonal therapy for prostatic cancer (1). Later, DES was also prescribed to prevent threatened abortions (2) and used as a postcoital contraceptive (1).

At present, this compound is mainly used as an alternative treatment to orchiectomy in patients with prostatic metastases because of the well known and understandable psychological reaction to castration (3). Thus, mini-doses of DES plus low-doses of cyproterone acetate produce a reversible medical castration (4). DES works by suppressing LH production and, indirectly by decreasing serum testosterone level.

However, DES has the potential to cause serious cardiovascular or thromboembolic complications (5). In order to reduce these adverse effects, the concentration of the drug must be minimized by means of controlled release delivery systems, among which cyclodextrin encapsulated drugs are perhaps the most promising (6).

Cyclodextrins are cyclic host molecules of cylindrical shape consisting of six, seven or eight glucopyranose units linked by glycosidic $\alpha(1 \rightarrow 4)$-type bonds which are formally named as α, β, and γ-cyclodextrins, respectively. All the polar hydroxyl groups are orientated towards the exterior, leaving the skeletal carbons and the ethereal oxygens within the cylinder. This steric configuration results in an apolar and hydrophobic interior, while the rims of the surrounding walls are hydrophilic (7). A wide range of organic and inorganic guest molecules can be accommodated in the hydrophobic cavity, forming inclusion complexes. These complexes are water solubles to a greater or lesser extent, depending on the ciclodextrins used and the drug complexed. They may be in solution or be precipitate as a microcrystalline powder, which are identified by different techniques: X-ray powder diffractometry, differential thermal analysis (DTA), IR spectroscopy or NMR studies (8). In this complexed state, these guest molecules exhibit differences in their physicochemical behavior, a property which has been exploited commercially and which is of special interest to the pharmaceutical industry (6). Cyclodextrins can be advantageously used to alter the properties of drug molecules, thus reducing their side effects, to modulate high peak plasma concentrations, and to diminish the adverse effects of rapid absorption (9).

Modified cyclodextrins, which have one or more branches of an α-D-glucopyranosil unit or a $(1 \rightarrow 4)$-α-D-glucan at the carbon 6 site of the glucose residues, have many advantages over the parent cyclodextrins. For example, they are highly soluble, both in water and in organic solvents, and enhance the solubility of otherwise water-insoluble compounds by complexation (10–13). Among them, 2-hydroxypropyl-β-cyclodextrins (HP-β-cyclodextrins) is one of the most widely used for its high solubility and low cost. These properties have been exploited commercially, especially in the pharmaceutical field (14,15).

In this paper, the *in vitro* formation of DES-cyclodextrins inclusion complexes was characterized using an enzymatic system (lipoxygenase). This enzyme oxidizes DES in the presence of H_2O_2 in aqueous medium (16). A study of this oxidation permits determination of the complexation constant between DES and cyclodextrins and evaluation of the release capacity of this drug in this system.

MATERIALS AND METHODS

Electrophoretically pure (17) soybean lipoxygenase (E.C. 1.13.11.12) Type V (646,000 Sigma units/mg protein) prepared by affinity chromatography (18) was purchased from Sigma, Madrid, Spain. DES was obtained from Janssen Chimica, Geel, Belgium. Cyclodextrins were kindly supplied by Amaizo, American Maize-Products Company, Hammond, Indiana. All other chemical used were of analytical grade.

The activity was followed spectrophotometrically in an Uvikon 940 (Kontron) or in a Hewlett Packard HP 8452A diode array at the absorption maximum of DES quinone (λ_{max} = 315 nm, ε_{315} = 29,600 M^{-1} cm^{-1}) (16).

Hydrogen peroxide and DES were freshly prepared every day. The H_2O_2 and lipoxygenase concentrations were calculated

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ABBREVIATIONS: DES, diethylstilbestrol; HP-β-cyclodextrins, hydroxypropyl-β-cyclodextrins, LOX, lipoxygenase.

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using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$ (19) and $\varepsilon_{280} = 160{,}000\ M^{-1}\ cm^{-1}$ (20), respectively.

Phase diagrams were carried out according to Higuchi and Connors (1965) (21) as follows: excess amounts of DES were added to aqueous solutions containing increasing concentrations of α-, β-, λ- and HP-β-cyclodextrins up to 100 mM (15 mM in the case of β-cyclodextrins, its solubility limit) in 10 mM phosphate buffer (pH 7.4) and shaken at 25°C for 3 days to reach equilibrium. The aqueous solutions were filtered through a 0.2 μm membrane filter and diluted in 80% ethanol-water. The DES concentration was spectrophotometrically determined ($\varepsilon_{240} = 13{,}000\ M^{-1}\ cm^{-1}$) and the filtrate absorbance was measured at 240 nm.

RESULTS

Lipoxygenase (linoleate oxygen oxidoreductase E.C. 1.13.11.12) (LOX) is a non-heme iron-containing enzyme that has two activities, dioxygenase and hydroperoxidase, both of which are associated with a single protein (13,16–18,22–25). This enzyme catalyzes the oxidation of DES to its corresponding quinone in the presence of H_2O_2 in buffer medium (16). This enzyme also catalyzes the oxidation of DES in the presence of cyclodextrins, producing a characteristic yellow color with two maxima, at 315 nm and 340 nm (Fig. 1A) corresponding to a DES quinone product previously described in the oxidation of DES by LOX in the absence of cyclodextrins (16). The formation of an isosbestic point at 260 nm and the graphic matrix analysis of the repetitive scan of Fig. 1A, using the method by Coleman *et al.* (26), demonstrated the presence of two kinetically related species, DES and DES-quinone (Fig. 1B).

The fact that the oxidation of DES by LOX can be measured in the presence of cyclodextrins, can be used to determine the complexation constant by means of the enzymatic method recently described by our group (27).

An experiment was performed in which LOX activity was determined with different cyclodextrin types at different concentrations, while the DES concentration was kept constant. A drastic decrease in LOX activity was observed as β-, HP-β- and γ-cyclodextrin concentration increased (Fig. 2 filled circles, filled squares and open squares, respectively). We suggest that this inhibitory effect on LOX activity is most probably due to DES entering into the hydrophobic cavities of these three types of cyclodextrins. The presence of α-cyclodextrins in the reaction medium, on the other hand, had no effect on the enzymatic activity (Fig 2, open circles) since its hydrophobic cavity is smaller than those of other cyclodextrins.

The linear relationship observed between this low concentration of cyclodextrins and the concentration of DES (Fig. 3, inset) corresponds to a 1:1 complex formation, as is well known in the cyclodextrins field since the work of Higuchi and Connors in 1965 (21). Assuming this, the equilibrium between DES and cyclodextrins can be expressed as:

$$DES_f + CD_f \overset{K_c}{\leftrightarrow} DESCD \quad (1)$$

where subscript f refers to the concentration of the free compound, and the complexation constant K_c is usually defined as:

$$K_c = \frac{[DESCD]}{[DES]_f[CD]_f} \quad (2)$$

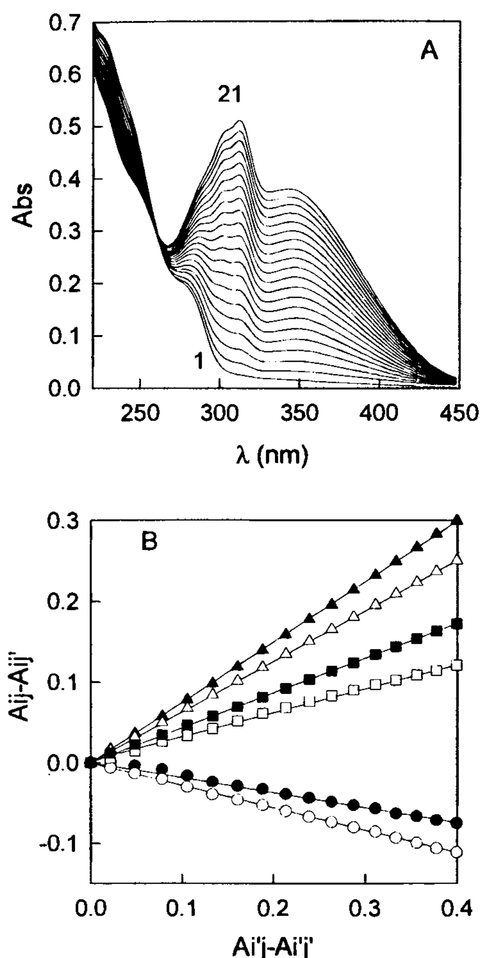

Fig. 1. (A) Oxidation of DES produced by lipoxygenase in the presence of H_2O_2 and cyclodextrins. The reaction medium at 25°C contained 10 mM phosphate buffer pH 7.4, 37 μM total DES, 100 μM total 2-HP-β-cyclodextrins, 125 μM H_2O_2 and 150 μM lipoxygenase. The scans were obtained every 30 s. (B) Coleman's graphical analysis for two absorbing species. In this analysis, A_{ij} is the absorbance at wavelenght *i* obtained during tracing *j*: *i'* = 310 nm, *j* = first trace, i = ● (220 nm), i = ○ (230 nm), i = □ (280 nm), i = ▲ (350 nm), i = △ (370 nm), i = ■ (390 nm).

Taking into account the mass balance

$$[DES]_t = [DES]_f + [DESCD] \quad (3)$$

$$[CD]_t = [CD]_f + [DESCD] \quad (4)$$

and Eq. 2, $[CD]_f$ and [DESCD] can be expressed as:

$$[CD]_f = \frac{[DES]_t - [DES]_f}{K_c[DES]_f} \quad (5)$$

$$[DESCD] = K_c[DES]_f[CD]_f \quad (6)$$

Then, substituting these last two equations into Eq. 3, the following quadratic relationship was obtained:

$$K_c[DES]_f^2 + ([CD]_tK_c - [DES]_tK_c + 1)[DES]_f - [DES]_t = 0 \quad (7)$$

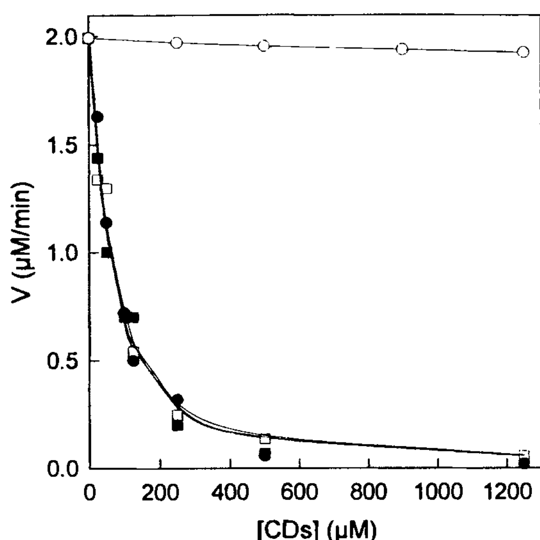

Fig. 2. Effect of different types of cyclodextrin concentration on DES oxidation by lipoxygenase. The reaction medium at 25°C contained 10 mM phosphate buffer pH 7.4, 50 μM DES, 0.125 mM H_2O_2, 39 nM lipoxygenase and increasing concentrations of (○) α-, (●)β-, (□) γ- and (■) HP-β-cyclodextrins, from 0 to 1.25 mM.

From this, $[DES]_f$ can be obtained:

$$[DES]_f = \left(\frac{-([CD]_tK_c - [DES]_tK_c + 1) + \sqrt{([CD]_tK_c - [DES]_tK_c + 1)^2 + 4K_c[DES]_t}}{2K_c}\right) \quad (8)$$

Considering that the enzyme works in a range of DES concentrations below K_M (due to its low aqueous solubility) and that free DES is the only form of substrate LOX that can be used, the reaction rate in the presence of cyclodextrins can be expressed as:

$$v = \frac{V_m}{K_M}[DES]_f \quad (9)$$

Substituting Eq. 8 into Eq. 9:

$$v = \frac{V_m}{K_M}\left[\frac{(-([CD]_tK_c - [DES]_tK_c + 1) + \sqrt{([CD]_tK_c - [DES]_tK_c + 1)^2 + 4K_c[DES]_t})}{2K_c}\right] \quad (10)$$

Eq. 10 shows a nonlinear relationship between v and $[CD]_t$, as in Fig. 2. Fitting the data by nonlinear regression using Sigma Plot (Jandel Scientific), values of 2.9×10^4 M^{-1}, 2.6 $\times$ 10^4 M^{-1} and 2.8×10^4 M^{-1}, for the K_c of β-, γ- and HP-β-cyclodextrins, respectively, were obtained.

These values are in agreement with the K_c calculated using the physical method of Higuchi and Connors (1965) (21) and their equation

$$K_c = \frac{\text{slope}}{[S_0](1 - \text{slope})} \quad (11)$$

where $[S_0]$ is the solubility of a compound in water (Fig. 3). The K_c values were calculated from the initial straight line portion of the phase solubility diagrams (Fig. 3, inset) where the cyclodextrins concentration are of the same order as in Fig. 2 and the stoichiometry of the complexes formed could be considered as 1:1.

In order to clarify whether or not the inhibition observed in Fig. 2 is due to the complexation of DES into cyclodextrins, the concentration of the free substrate at each point was calculated using the previously obtained K_c values and Eq. 8. The values of free substrate were seen to decrease as cyclodextrins concentration increased (Fig. 4). This Fig. 4 is similar to Fig. 2, in which the LOX activity is represented vs. cyclodextrin concentration, indicating that free DES is the only form of substrate that LOX can use. Moreover, when free DES concentration data were represented vs. LOX activity, a linear increase in enzymatic activity was observed (Fig. 4, inset). This result indicates that the enzyme was working at DES concentrations below K_M and confirms the fact that LOX only works with free substrate and not with complexed DES, because the total DES concentration was always the same.

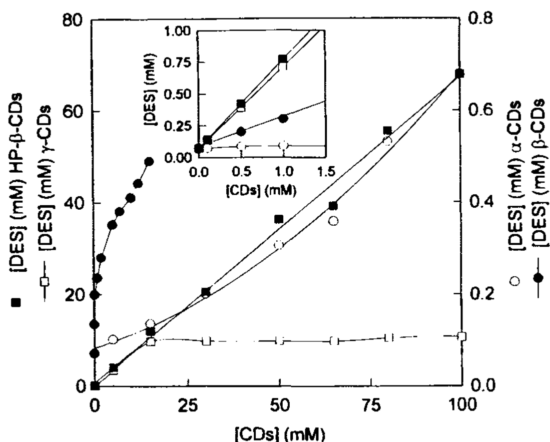

Fig. 3. Phase solubility diagram of DES and different types of cyclodextrins in phosphate buffer 10 mM pH 7.4 at 25°C (○) α-, (●)β-, (□) γ- and (■) 2-HP-β-cyclodextrins. Inset: Enlargement of phase diagrams from 0 to 1.5 mM of cyclodextrins.

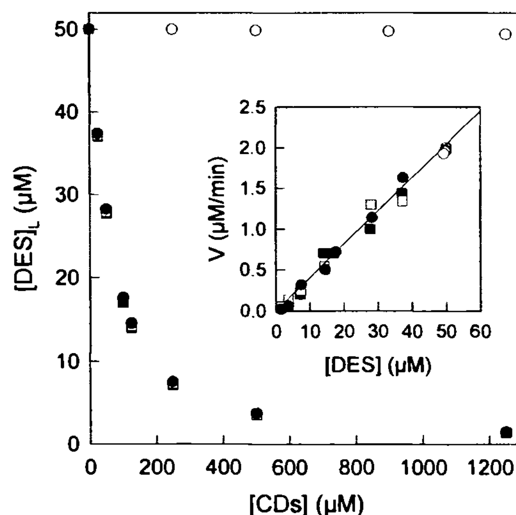

Fig. 4. Effect of different types of cyclodextrin concentration on free DES concentration. Free DES concentration in different types of cyclodextrins replotted from Fig. 2 using Eq. (8): (○) α-, (●) β-, (□) γ- and (■) 2-HP-β-cyclodextrins. Inset: Effect of free DES concentration on the activity of lipoxygenase.

Table I. Solubility of DES in Aqueous Medium by Complexation with Cyclodextrins and Hydroperoxidase Activity of Lipoxygenase on Each Disolutions

	H_2O	α-CDs		β-CDs[a]		γ-CDs		2-OHP-β-CDs	
		1 mM	100 mM	1 mM	15 mM	1 mM	100 mM	1 mM	100 mM
$[DES]_t$ (mM)	0.071	0.09	0.68	0.24	0.49	0.71	10.64	0.77	67.86
Activity[b] (μM/min)	3.34	3.25	3.09	3.35	3.42	3.19	3.12	3.08	3.28

[a] The aqueous solubility limit of β-cyclodextrins is 15 mM.
[b] The activity was measured at 71 μM of free DES concentration.

Thus, LOX activity acts as an indicator of the free DES concentration present in the reaction medium, demonstrating that cyclodextrins can be used to decrease the free DES level while the total DES concentration remains constant, or to increase total DES concentration while free DES concentration remains constant.

One interesting property of cyclodextrins is that they can be saturated with DES and used to add high levels of drug to the reaction medium, up to 956 times compared with water in the case of HP-β-cyclodextrins (Table I), while the free DES concentration remains constant. As shown in Table I, LOX activity was independent of the total DES concentration, indicating that the free DES concentration keeps constant and coincides with the aqueous solubility limit of the drug, independently of the type and concentration of cyclodextrins used.

Depending on the concentration and type of cyclodextrin used, different concentrations of total DES may be added to the reaction medium, whereas the free DES concentration remains constant. Thus, Table I shows that HP-β-cyclodextrins are capable of increasing the total DES concentration in aqueous solution 956 times, although the increases are lower with β- and γ-cyclodextrins, despite the similar K_c value.

The DES-cyclodextrin complex could be considered as a DES repository that is converted to free DES as this drug disappears from the reaction medium. This effect can be observed in Fig. 5, which shows how LOX activity remains linear with time depending on the DES remaining in the reaction medium (Fig 5, trace b), whereas the initial rate depends only on the free DES concentration. Thus, DES is liberated progressively from cyclodextrins in accordance with its transformation by the enzyme.

In conclusion, this paper clearly shows that DES can be included in cyclodextrins and that DES-cyclodextrins might be a very suitable drug carrier for delivering DES without side effects, since it controls the free DES concentration according to the equilibrium constant calculated in this paper.

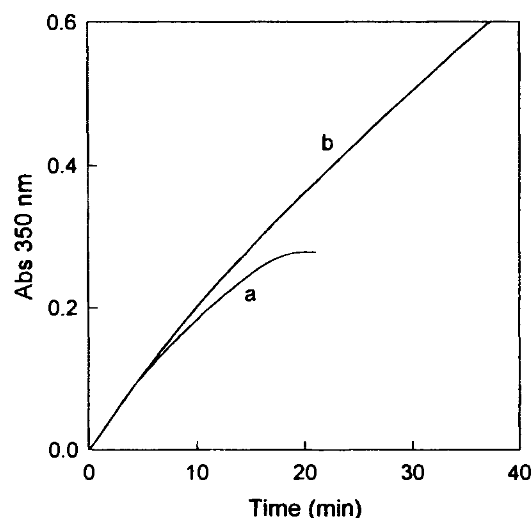

Fig. 5. Time course of oxidation of DES by lipoxygenase in the absence (a) and presence (b) of cyclodextrins. The reaction medium at 25°C contained 10 mM phosphate buffer pH 7.4, 0.125 mM H_2O_2, 150 nM lipoxygenase and (a) 25 μM DES, (b) 25 μM free DES, but 1 mM total DES.

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