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Fluorometric Determination of Association Constants of Three Estrogens with Cyclodextrins

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It was found that the fluorescence intensity in solutions of three estrogens—estradiol, ethinyloestradiol, and estriol—increased upon the addition of β- or γ-cyclodextrin, the effect being greater with the first ligand. These changes in fluorescence intensity were used for the determination of association constants of the compounds with the two cyclodextrins. An iterative procedure was used to calculate the concentrations of free (uncomplexed) ligand, which are needed for the calculation of the association constants. These values were compared with the constants previously determined by HPLC. The fluorometric association constants with β-cyclodextrin were lower than the corresponding HPLC values, whereas for the complexes with γ-cyclodextrin the results of both methods coincide.

KEY WORDS: Fluorescence; association constants; estrogens; β-cyclodextrin; γ-cyclodextrin.

INTRODUCTION

Cyclodextrins (CDs) form host–guest complexes with a number of molecules. Fluorescence quantum yield is one of the physicochemical properties of guest molecules which can be affected upon complex formation[1–6]. It was reported that one class of steroids—the 4,6,8(14)-triene compounds, highly fluorescent in aqueous solutions—shows a drastic decrease in fluorescence intensity upon the addition of β-CD to their solutions.[6] Now it appears that the fluorescence intensity of other steroid compounds, estrogens, having a phenolic ring, increases upon complexation with β-CD and γ-CD. Such a change in the fluorescence intensity can be used for the determination of the association constants of complexes of the three compounds with both CDs. The structure of the three studied estrogens is shown in Fig. 1. The association constant (K_a) values of these compounds with β- and γ-CDs were measured previously in the same medium and at the same temperature by high-performance liquid chromatography.[7] It seemed interesting to determine these constants by another method and to compare the results. In the present study we investigated changes in fluorescence intensity of three estrogens—estradiol, ethinyloestradiol, and estriol—due to complexation with β- and γ-CD, and we determined the association constants of these complexes.

EXPERIMENTAL

Fluorescence spectra were measured with a Shimadzu RF-5000 spectrofluorometer, equipped with a thermostatically controlled cell compartment. The spectra were measured at 35°C. The excitation wavelength was 280 nm; the molar absorption coefficient of the compounds at 280 nm was about 2200. The maximum value at 310–311 nm or the integrated fluorescence spectrum was taken as a measure of the fluorescence intensity. β-CD was purchased from Sigma Chemical Co. (St Louis, MO). γ-CD was from Merck (Darmstadt, Germany). The water content in CDs was determined by the

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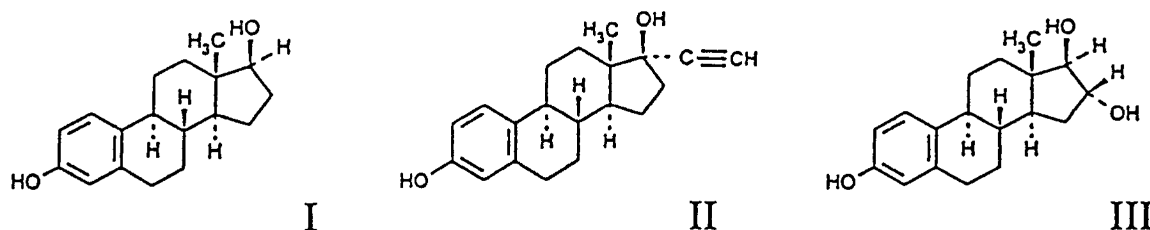


Fig. 1. Formulas of estrogens: I, estradiol; II, ethinyloestradiol; III, estriol. Registry Numbers: estradiol, 50-28-2; ethinyloestradiol, 57-63-6; estriol, 50-27-1.

Karl-Fisher method.[8] Solutions of fluorophores and stock solutions of CDs (about 10 mM) were prepared fresh each day.

Fluorescence Titration

Two mililiters of an estrogen solution (0.8×10^{-5}–2×10^{-5} M) in methanol/water (20/80, v/v), in a fluorescence cell with a Teflon stopper and magnetic stirrer, was titrated with successive additions of a CD dissolved in the same estrogen solution at a concentration of about 10 mM. The portions added were in the range of 10–100 µl. Final concentrations of β- and γ-CD ranged from 0 to 1.3 mM. After each titration the fluorescence spectrum as well as the intensity at the maximum wavelength was recorded as a function of the ligand concentration. In the case of β-CD the measurements had to be made quickly after dissolving β-CD, because after about 1 h the complex precipitate should be observed in the stock (concentrated) solution of β-CD.

The CDs used should be transparent at 280 nm but instead they showed a weak absorption, which, for β-CD at a concentration of 6.3 mM and for γ-CD at a concentration of 10 mM, was 0.045 (path length, 1 cm). Due to this absorption the estrogen fluorescence intensity was lowered slightly. Suitable corrections were therefore made, according to the relation $F = 2.3\ I_0 \eta \varepsilon l c$, where F is the fluorescence intensity, η is the fluorescence quantum yield, ε is the molar absorption coefficient at the excitation wavelength, l is the path length, and c is the molar concentration.[9] If the light intensity in the complex solution is lowered due to CD absorption, F is also lowered in the same proportion. So for every concentration of CD the measured F values were corrected. For example, for the 1.3 mM β-CD solution, 2% of the incident light intensity was absorbed by β-CD. The corresponding F value was divided by 0.98 (for lower CD concentrations, the correction factors were nearer to unity).

Job's Analysis

For determination of the complexes' stoichiometry, equimolar solutions (2×10^{-4} M) of ethinyloestradiol (E) and β- or γ-CD were prepared and mixed to produce two series of seven solutions containing, in succession, 2, 3, 4, . . . , 8 ml of E and 8, 7, 6, . . . , 2 ml of solvent (first series) or CD (second series). The total volume of each solution was 10 ml. The fluorescence intensity of all solutions was measured, and from the value for each solution in the second series, F_{E+CD}, the value for the corresponding member in the first series, F_E (that would be observed in the absence of complexation), was subtracted.

RESULTS AND DISCUSSION

The addition of β- or γ-CD to estradiol, ethinyloestradiol, or estriol solutions in methanol/water (20/80, v/v) resulted in an increase in fluorescence intensity. The emission maximum was slightly shifted from 311 to 310 nm in the case of β-CD but was unchanged for complexes with γ-CD. Fluorescence intensity was measured at the maximum wavelength, 310–311 nm, or as an integrated whole spectrum—the results were virtually the same. The fluorescence spectra of estradiol solutions containing varying concentrations of β-CD are shown in Fig. 2. For comparison of the influence of both ligands the fluorescence spectra of ethinyloestradiol with the addition of β- and γ-CD are shown in Fig. 3. Ligand concentrations were chosen which produced the same fractions of the complexed substrate in both cases. These fractions were, from top to bottom, 0.8, 0.7, 0.6, 0.4, and 0. It can be seen that the fluorescence intensity is more enhanced in the complexes with β-CD than in the complexes with γ-CD. This fact, together with the emission maximum wavelength shift in the case of β-CD, is probably due to the tighter complex structure and stronger intermolecular interactions in the case of β-CD, which has a smaller cavity than γ-CD. Complexation of

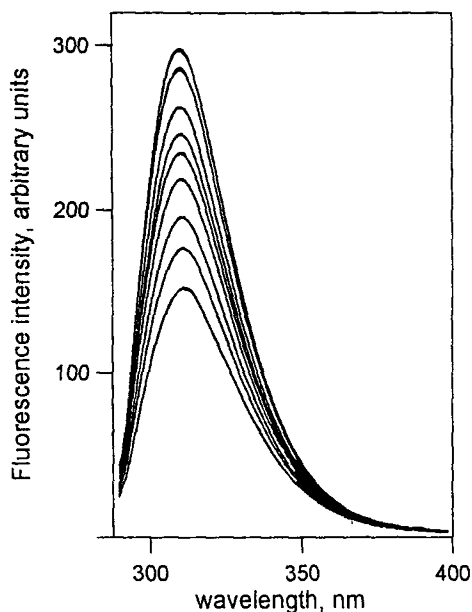


Fig. 2. Fluorescence spectra of estradiol in methanol/water (20/80, v/v) at 35°C, with the addition of β-cyclodextrin at (from top to bottom) 1.7, 1.2, 0.69, 0.47, 0.35, 0.24, 0.12, 0.06, and 0 m*M*.

the compounds also influenced their absorption spectra, but to a lesser degree than it did their emission spectra. The absorption spectra of ethinyloestradiol alone and with the addition of 0.7 m*M* β-CD and 0.4 m*M* γ-CD are shown in Fig. 4. In both cases the absorption maximum is slightly shifted (about 1 nm) to longer wavelengths, and in the case of β-CD the absorbance is increased by a few percent. This change in absorbance upon the addition of β-CD, though not large, is a reason to think that the enhancement of fluorescence intensity not only is connected with the decrease in the rate constant of radiationless deactivation of the excited state due to the more rigid local environment of a complexed molecule, but also can be due partially to the increased radiative transition moment between the excited and the ground states.

If the host:guest ratio in the complex is 1:1, the relation analogous to the Benesi-Hildebrand one holds for the dependence of the increment of fluorescence intensity ΔF and CD concentration [CD]:

$$\frac{F_o}{\Delta F} = \frac{\eta_o}{(\varepsilon/\varepsilon_o)\,\eta - \eta_o} + \frac{\eta_o}{(\varepsilon/\varepsilon_o)\,\eta - \eta_o} \cdot \frac{1}{[\mathrm{CD}]\,K_a} \quad (1)$$

where F_0 is the fluorescence intensity without CD, η_0 and η are the fluorescence quantum yields without and with CD, and ε_0 and ε are the molar absorptivities without and with CD. Thus an estimate of K_a can be obtained from a plot of $F_0/\Delta F$ vs 1/[CD] by dividing the y intercept by the slope. It can be proved that F_0 and ΔF can

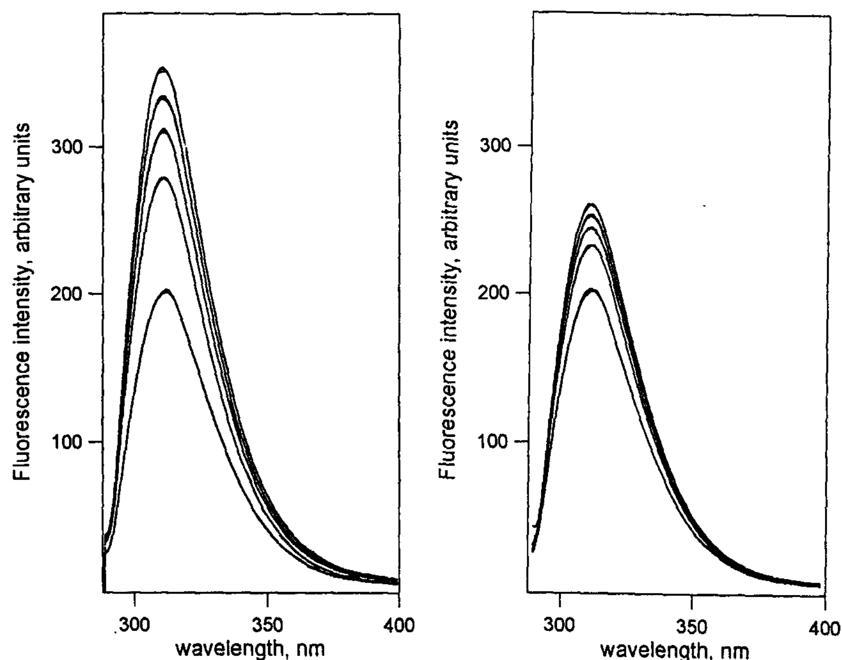


Fig. 3. Enhancement of the fluorescence intensity of ethinyloestradiol by the addition of cyclodextrins Left: Concentrations of β-cyclodextrin of (from top to bottom) 0.7, 0.4, 0.2, 0.1, and 0 m*M*. Right: Concentrations of γ—cyclodextrin of (from top to bottom) 0.4, 0.2, 0.1, 0.06, and 0 m*M*. Fractions of the complexed substrate for both cyclodextrins were (from top to bottom) 0.8, 0.7, 0.6, 0.4, and 0.

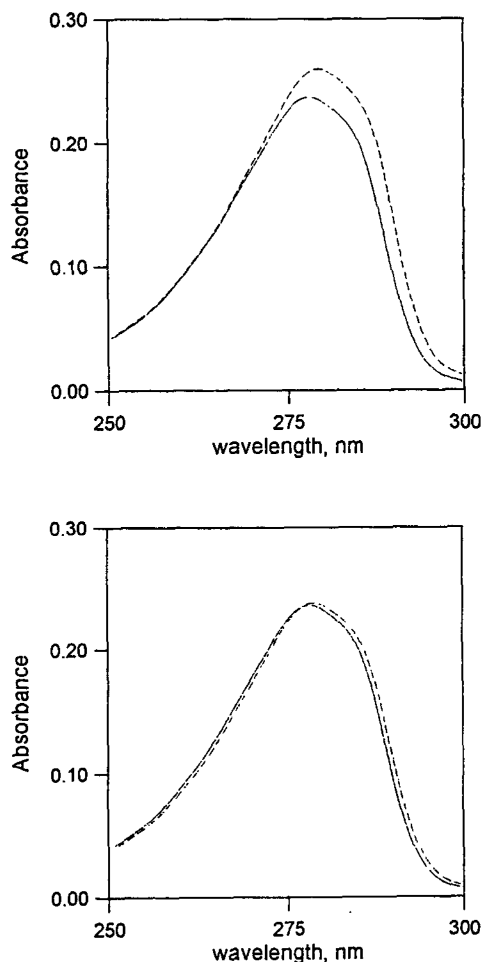


Fig. 4. Absorption spectra of ethinyloestradiol in methanol/water (20/80, v/v) at 35°C (lower curves) and with the addition of (top) 0.7 m*M* β-CD and (bottom) 0.4 m*M* γ-CD (upper curves). The fraction of the complexed substrate in both CDs solutions was 0.8.

be measured in the absence or presence of a quencher, e.g., oxygen in the solution; even if the effect of quenching is different for quantum efficiencies of free and complexed ligand, in Eq. (1) the terms containing quantum efficiencies are canceled.

Nevertheless, one encounters a difficulty here, namely, [CD] is the concentration of free (uncomplexed) CD, which is not known. In references devoted to the determination of K_a, it is usually assumed that a large ligand excess relative to the substrate is employed so the total concentration of CD can be substituted in Eq. (1) instead of the free CD concentration. This assumption is not always justifiable. In the present work the substrate concentration was 0.8×10^{-5}–2×10^{-5} *M* and could not be much less, because the absorption coefficient of the longest-wavelength band was low and the quantum yield was not very high either (0.20 in an ethanolic solution at room temperature[10]). On the other hand, a CD concentration range was chosen that would produce about 15–90% of the compound in the complexed form in the steroid solutions. These required concentrations of CDs were calculated according to the equation for the binding isotherm,[11]

$$f = \frac{K_a[L]}{1+K_a[L]} \qquad (2)$$

where f is the fraction of complexed substrate, K_a is the association constant, and [L] is the concentration of ligand (here CD), and ranged from 5×10^{-5} to 1.3×10^{-3} *M*. For example, for estradiol at a total β-CD concentration of 5×10^{-5} *M*, $f = 0.156$, and the concentration of the complex formed is 3×10^{-6} *M*. So about 6% of the total β-CD concentration is bound to estradiol. To find the concentrations of free CDs when association constants are not known *a priori*, an iterative procedure was used. First, the total CD concentrations were substituted in Eq. (2), then a tentative value of K_a was found. Next the concentrations of free CD for every ith total concentration of CD, $[CD]_i$, were calculated in the first approximation using the estimated value of K_a according to the following equation:

$$K_a = \frac{x_i}{([S] - x_i)\,([CD]_i - x_i)} \qquad (3)$$

where [S] is the total substrate concentration; x_i, the concentration of the complex formed; and $([CD]_i - x_i)$, the concentration of free CD. Then the values of $1/\Delta F$ were regressed against a set of new values $([CD]_i - x_i)$ instead of $[CD]_i$, and the procedure was repeated until no difference between subsequent values of K_a was found. The last (iterated) values are given in Table I, together with K_a values determined previously chromatographically.[7] The plot of $F_0/\Delta F$ against 1/[CD] for estradiol is shown in Fig. 5. Other plots were also rectilinear: typical values of the regression coefficients were 0.999.

Linearity of Benesi–Hildebrand plots is an indication that the complexes stoichiometry is 1:1. To strengthen the conclusion, Job's analysis, or the method of continuous variation,[12] was used for ethinyloestradiol—the compound for which the association constants and the fluorescence intensity changes were the largest. Plots obtained with β- and γ-CD are given in Figs. 6a and 6b. They were produced by fitting parabolic curves to the experimental points by the least-squares method. Maximum values of Job's curves were found at 0.49 for β-CD and 0.47

Table I. Association Constants of Three Estrogens with β- and γ-Cyclodextrin in Methanol/Water (20/80 v/v) at 35°C, Measured Fluorometrically, with Their Standard Deviations[a]

Compound	K^a (M^{-1})	
	β-CD	γ-CD
Estradiol	3700 ± 700 (6800)[a]	6350 ± 300 (7100)
Ethinyloestradiol	5600 ± 500 (7600)	9300 ± 1400 (10600)
Estriol	3200 ± 400 (4700)	3200 ± 600 (3200)

[a]Values in parentheses are the corresponding constants measured by HPLC [7].

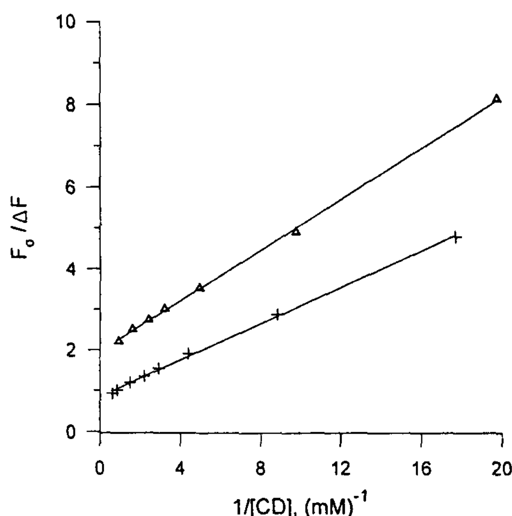
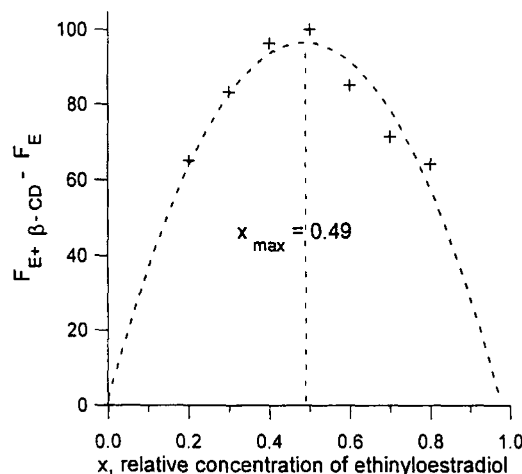


Fig. 5. Plots of the reciprocal increment of fluorescence intensity $F_0/\Delta F$ *vs* the reciprocal concentration of free cyclodextrin: (+) β-cyclodextrin and (Δ) γ-cyclodextrin, according to Eq. (1).



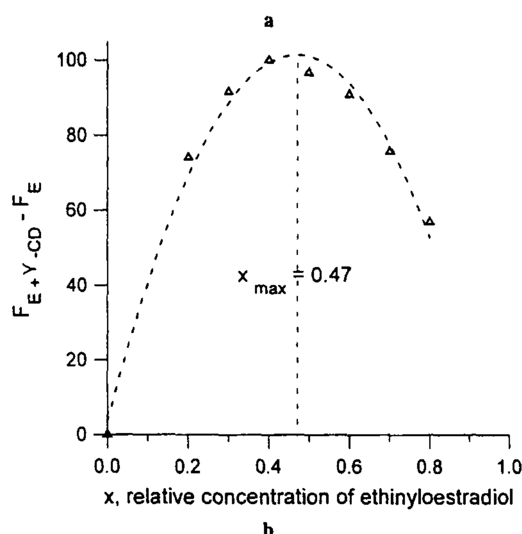


Fig. 6. Job's curves plotted for (a) ethinyloestradiol and β-cyclodextrin and (b) ethinyloestradiol and γ-cyclodextrin.

for γ-CD, which is near the theoretical value of 0.5 expected for complexes of stoichiometry 1:1.

Table I shows that the values of association constants with β-CD determined fluorometrically are lower than the same values obtained by means of HPLC, the fluorometric values amounting to 54–74% of the chromatographic ones. The corresponding values for γ-CD determined by both methods coincide within their experimental error.

The chromatographic constants characterize the reaction of complex formation by the substrate molecules in their ground state. If the complexation equilibrium is achieved sufficiently quickly, monitoring of the fluorescence intensity provides us with the opportunity to study complexation behavior in the substrate excited state. So the difference between the association constants determined by the two methods can be due to the different complexation degrees in both states.[1] It is also possible that the difference is due to other reasons, e.g., any systematic error connected with one or both methods. To elucidate the question it would be useful to make similar measurements of the association constants by fluorometric and HPLC (or other) methods with other compounds.

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