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Influence of the Structure of Steroid Hormones on Their Association with Cyclodextrins: A High-Performance Liquid Chromatography Study

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Abstract. The association constants of fourteen steroid hormones with β- and γ-cyclodextrin were measured in methanol–water (20 : 80 v/v) at 35 °C using the chromatographic Hummel–Dreyer method. It was found that the greatest influence on the association constants is the structural features of ring A of these compounds but the substituents of ring D also alter the complex stability to an appreciable degree. The measured association constants were considerably greater than the corresponding values measured previously in the medium containing more methanol (45 instead of 20%).

Key words: Steroid hormones, steroids, β-cyclodextrin, γ-cyclodextrin, complexation, association constant, HPLC.

1. Introduction

Steroid hormones are a class of compounds which are known to form inclusion complexes with cyclodextrins. The complexes are interesting from the point of view of intermolecular interactions. Investigation of these interactions and the structure of the complexes requires the determination of the stoichiometry of the inclusion and the association constants. Most techniques used for this purpose are mentioned in Ref. [1], but one can add to the list the transport method [2] (applicable to compounds insoluble in water) and a polarographic method [3, 4]. One of these methods is high-performance liquid chromatography (HPLC), which is a convenient tool for studying the equilibria taking place in solutions, especially in mixtures of water and an organic modifier. Using HPLC, one can follow the formation of complexes through the decrease of the retention time of the substrates after addition of the complexing agent to the eluent [5, 6] or by the Hummel–Dreyer method (see Section 2.3: Procedure).

The aim of this work was to determine association constant (K_a) values of complexes of a wider range of steroid hormones with β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD) in order to throw some light on the contribution of various structural details to the stability of the complexes. The method used was that

of Hummel–Dreyer. This method was previously used for the determination of association constants of four estrogens (estradiol, ethinyloestradiol, estrone and estriol) with cyclodextrins in a methanol–water (45 : 55 v/v) medium [7].

2. Experimental

2.1. MATERIALS

β-CD was purchased from Sigma Chemical Co. (St. Louis, USA). γ-CD was from Merck (Darmstadt, Germany). The water content of the CDs was determined by the Karl–Fischer method. The formulas of fourteen steroids whose complexation by β- and γ-CD was investigated are given in Figure 1, together with their common names and CAS registry numbers. The conventional representation of the steroid ring system and the carbon atom numbers are given in Figure 2. For the stock solutions a weighed portion of a steroid (3–8 mg) was dissolved in about 20 mL of methanol and a fourfold volume of water was added. The solution was made up to 100 mL with methanol–water (20 : 80). This procedure was used because of the low solubility of this class of compounds (except estriol, bethamethasone phosphate and dexamethasone phosphate, which were dissolved directly in methanol–water (20 : 80). To produce a mobile phase, the stock solution was diluted 5 or 10 times with methanol–water (20 : 80). The final concentration was in the range 0.02–0.04 mM. In order to ensure the greatest possible precision and repeatability in producing the methanol–water (20 : 80) mixtures, both components were weighed and the volumes calculated using the known density of the solvents. For calibration the stock solutions were diluted 2.5 or 5 times, so their concentrations were twice those of the mobile phases. To produce solutions of CDs for injection, the weighed portions of β- or γ-CD were dissolved directly in the mobile phase (with gentle heating in the case of β-CD).

2.2. APPARATUS

The LC system used was a Shimadzu pump (LC-10AS), oven (CTO-10AC), UV detector (SPD-10A), and integrator (R6A). For all compounds, except for betamethasone phosphate and dexamethasone phosphate, three columns were used: Hypersil SAS (3 μm, 50 $\times$ 4 mm i.d.), Eurospher 100 DIOL (7 μm, 250 $\times$ 4.0 mm i.d.), and LiChrospher 100 DIOL (5 μm, 120 $\times$ 4.0 mm i.d.). For betamethasone and dexamethasone phosphates the retention time on these columns was too low and another column was used: Waters μ-Bondapak-Phenyl, 300 $\times$ 3.9 mm i.d. Detection was effected at 280 nm (compounds **I**–**IV**) or 240 nm (others), near the absorption maxima of the steroids. A temperature of 35 °C was chosen in order to compare the present results in methanol–water (20 : 80) with the previous ones in methanol–water (45 : 55) [1, 7].

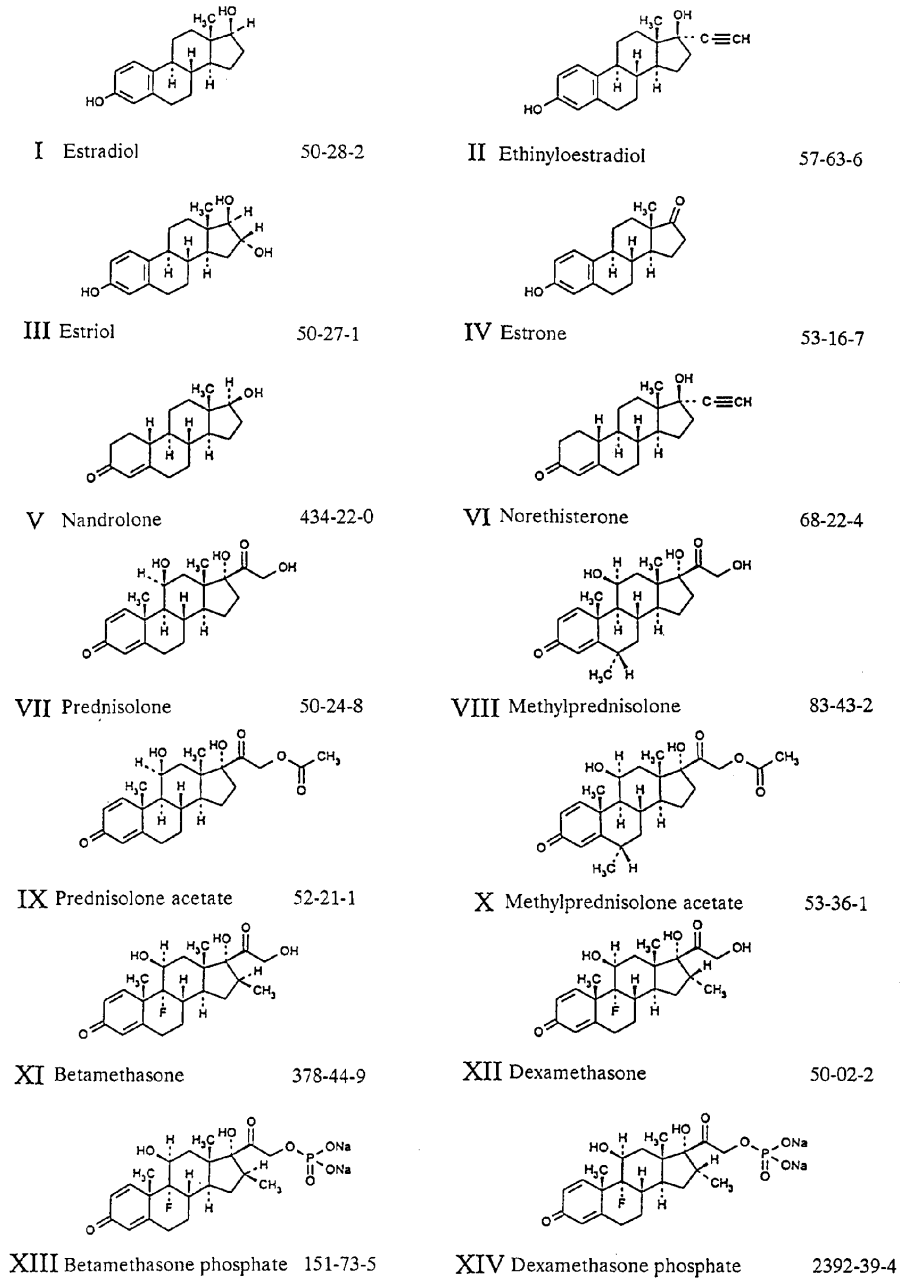

Figure 1. The formulas and CAS registry numbers of the fourteen investigated steroids whose complexation was investigated.

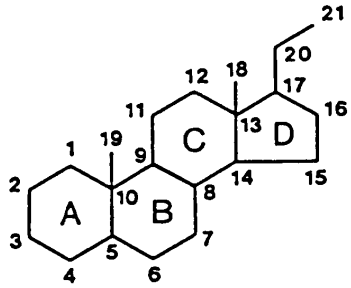

Figure 2. The conventional representation of the steroid ring system, showing the letters used to designate the rings and the numbers used to identify carbon atoms.

2.3. PROCEDURE

Association constants were measured by the Hummel–Dreyer method, which is based on the following procedure [7–12]. A column is equilibrated with an eluent carrying a solute molecule (S). A sample of a complexing agent (here CD) is prepared in the eluent solution and applied to the column. If sufficient complexation occurs and if CD and complex S–CD move faster than S, the chromatogram will contain the positive peak of S–CD and, behind it, at the elution position of a free molecule S, a negative peak that corresponds to the amount of S consumed to form S–CD. The height of the horizontal baseline corresponds to the concentration of S in the eluent. The area of the positive (or negative) peak is a measure of the concentration of the complex formed. The concentration of CDs in the solutions for injection were chosen such that about half of the compound was in the complexed form. These required concentrations of CDs were calculated using the binding isotherm equation [13]:

$$f = \frac{K_{\mathrm{a}}[\mathrm{L}]}{1 + K_{\mathrm{a}}[\mathrm{L}]} \tag{1}$$

where f = the fraction of complexed substrate; K_{a} = association constant; and [L] = concentration of free complexing agent (CD).

Using the value f = 0.5, the error in determination of K_{a} is minimized. (It is not much greater when working in the range of $f \approx 0.2$–0.8).

To calculate the amount of complexes formed the positive peaks were used. Association constants were calculated according to the equation:

$$K_{\mathrm{a}} = \frac{Q_{\text{S-CD}}}{[\mathrm{S}](Q_{\mathrm{CD}} - Q_{\text{S-CD}})} \tag{2}$$

where $Q_{\text{S-CD}}$ = total amount of the complex; Q_{CD} = total amount of CD applied; [S] = concentration of the steroid in the eluent.

Equation (2) is valid if the complex stoichiometry is 1 : 1. The Hummel–Dreyer method was also used for the determination of the complex stoichiometry. If a 1 : 1

complex is formed, then according to Equation (2), the application to the column of a given amount, Q_{CD}, of CD should produce twice that amount of the complex than after application of one half of this portion, $Q_{CD}/2$. In the case of the complex stoichiometry 1 : 2, S–$(CD)_2$, the ratio of the complex formed after application of Q_{CD} and $Q_{CD}/2$ is greater than 2 and depends on the individual values of Q_{CD} and the amount of the complex formed (S–$(CD)_2$).

The amount of complex formed was calculated from the positive peaks. Spectrophotometric measurements showed that in the case of compounds having a phenolic ring (**I**–**IV**) the absorbances of the complexes at 280 nm were about 7% higher than those of the free steroid compounds. So the areas of the positive peaks should be greater than the areas of the negative peaks by the same factor. Nevertheless the areas of these peaks are approximately equal, so corrections due to the higher absorbances of complexes were not introduced. But in the case of compounds **VII**–**XIV** with a dienone structure in ring A, the absorbances of the complexes at 240 nm were lower than those of the parent compounds and, correspondingly, the areas of the positive peaks were lower than those of the negative peaks. In the case of compounds **VII**–**X**, the absorbances of the complexes were about 12% lower for β-CD and γ-CD; in the case of compounds **XI**–**XIV** they were about 12% lower in the case of γ-CD but 20% in the case of β-CD. So the amounts of complexes formed calculated from the positive peaks were divided respectively by 0.88 or 0.80.

3. Results and Discussion

Typical chromatograms recorded for norethisterone (**VI**) are shown in Figure 3. The upper curve (a) was obtained after injection of an excess of the compound to the column eluted with a methanol/water (20 : 80) mobile phase containing 0.022 mM of norethisterone. Curve b was recorded after the injection of β-CD dissolved in the mobile phase.

For all the complexes studied their stoichiometry was investigated using the procedure described in the experimental part, e.g. by comparison of the amounts of the complex formed after injection of a given amount of CD, and of one-half of this portion. In all cases the ratio of the amounts of complex formed was near to 2 (the average value for all compounds being 2.0 for both CDs). This is a rationale for assuming that the investigated steroid hormones form complexes with β- and γ-CD of 1 : 1 stoichiometry.

The association constants of all the steroids investigated with β- and γ-CDs calculated assuming a 1 : 1 complex stoichiometry are given in Table I. For most of the complexes (23 of 28) the values were measured with a precision of about 10%; with the corrections due to the different absorbances of the complex and the parent compound the overall precision is about 15%. The complex stoichiometry and association constants could be also determined using the Scatchard plots [10, 12]. This procedure was followed for estriol, and the results are plotted as the

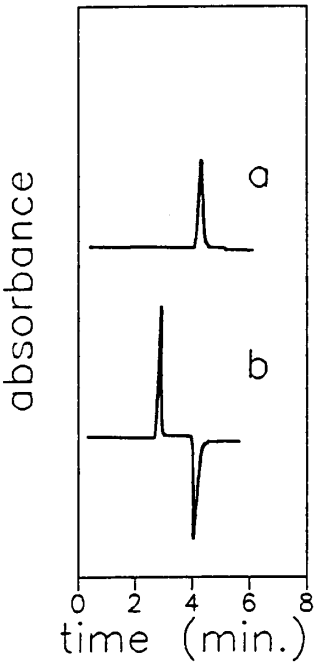

Figure 3. Typical chromatograms obtained with 0.022 mM norethisterone (**VI**) in the mobile phase (methanol–water 20 : 80 v/v): (a) corresponding to the injection of an excess of **VI**: (b) corresponding to the injection of β-CD dissolved in the mobile phase. Column used was Eurospher 100 DIOL 7 μm, 250 $\times$ 4 mm i.d.

dependence of $\bar{r}$/[S], where $\bar{r}$ is the ratio of the amount of estriol bound to the CD concentration, vs. $\bar{r}$ (Figure 4). From the slope and intercept of the plots the value of n (number of steroid molecules bound to one CD molecule) was calculated as 0.86 $\pm$ 0.12 for β-CD, and 0.92 $\pm$ 0.09 for γ-CD. The corresponding values for K_{a} were 5400 and 3500 ($\pm$10%) and agree within experimental error with the corresponding values determined with the same procedure as for other compounds: 4700 and 3200 (see Table I).

From the inspection of the data in Table I it can be concluded that the structural features of ring A have a great influence on the stability of the complexes. Two pairs of steroids – estradiol (**I**)–nandrolone (**V**) and ethinyloestradiol (**II**)–norethisterone (**VI**) –differ only in this ring property: in estradiol and ethinyloestradiol this ring is a phenolic one, unionized in neutral solution, because phenol is a very weak acid. In nandrolone (**V**) and norethisterone (**VI**) there is one double bond conjugated with a keto group. Both K_β and K_γ are greater for the compounds with a phenolic A ring: the ratio of the K_β values is 5 for the first pair and 8 for the second. The corresponding ratio for K_γ is 3 and 2, respectively. So it can be seen that the substitution of the phenolic ring with cyclohexenone has a greater influence on the

Table I. Association constants of the steroids with β-cyclodextrin (K_β) and with γ-cyclodextrin (K_γ) measured in methanol–water (20 : 80 v/v) at 35 °C with their standard deviations. Number of replicates are given in parentheses.

		K_β (M^{-1})	K_γ (M^{-1})
I.	Estradiol	6830 ± 680 (7)	7100 ± 400 (7)
II.	Ethinyloestradiol	7600 ± 770 (5)	10600 ± 960 (5)
III.	Estriol	4700 ± 400 (5)	3200 ± 370 (5)
IV.	Estrone	3100 ± 600 (6)	2550 ± 230 (6)
V.	Nandrolone	1300 ± 100 (6)	2300 ± 80 (6)
VI.	Norethisterone	940 ± 70 (6)	5300 ± 230 (6)
VII	Prednisolone	560 ± 50 (4)	980 ± 60 (4)
VIII.	Methylprednisolone	230 ± 15 (3)	1000 ± 80 (3)
IX.	Prednisolone acetate	570 ± 30 (30)	1370 ± 60 (5)
X.	Methylprednisolone acetate	230 ± 20 (4)	1330 ± 80 (4)
XI.	Betamethasone	800 ± 100 (7)	3150 ± 200 (7)
XII.	Dexamethasone	700 ± 40 (5)	3600 ± 200 (5)
XIII.	Betamethasone phosphate	350 ± 70 (3)	1500 ± 350 (3)
XIV.	Dexamethasone phosphate	400 ± 100 (3)	2000 ± 500 (4)

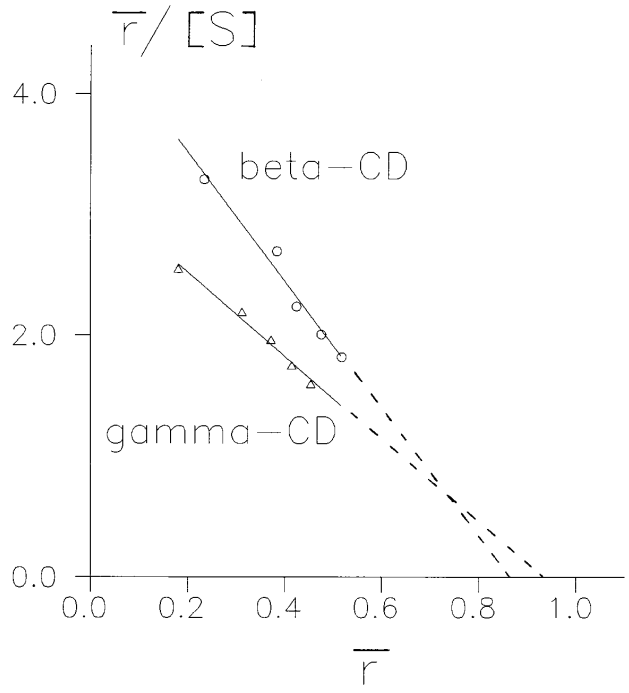

Figure 4. Scatchard plots obtained for the interaction of estriol with β- and γ-CD.

stability of complexes with β-CD than with γ-CD. It is interesting if this effect is due to steric effects connected with the shapes of ring A in both cases.

The second effect found is the lowering of the association constant for complexes of methylprednisolone (**VIII**) and methylprednisolone acetate (**X**) with β-CD as compared to the complexes of prednisolone (**VII**) and prednisolone acetate (**IX**). The former two compounds differ from the latter in having a methyl group attached to carbon 6 of ring B. This methyl group must be a serious steric hindrance for the entry of ring A into the interior of β-CD. In the case of complexes with γ-CD, which has a larger cavity, this effect was not found.

Predisolone (**VII**), prednisolone acetate (**IX**), betamethasone (**XI**) and dexamethasone (**XII**) do not have large association constants with β-CD. For nandrolone (**V**) and norethisterone (**VI**), which do not have a methyl group attached to carbon 10 nor an OH group at carbon 11, these constants are greater: one or both of these groups also make it difficult to complex these compounds with β-CD (but it is also possible that the presence of two double bonds in nandrolone (**V**) and norethisterone (**VI**) can also influence the complexation).

Nevertheless the properties of ring D, which should not be in contact with β-CD, if the complexation site is ring A, also influence the complex stability both with β-CD and with γ-CD. This is readily seen in the series of compounds whose structure is identical except for the ring D substituents: estradiol (**I**), ethinyloestradiol (**II**), estriol (**III**) and estrone (**IV**). The effect is even more pronounced with γ- than β-CD.

Inspection of Table I also reveals that esterification of the OH group at carbon 21 with acetic or phosphoric acid has a different effect on the stability of the complexes. The association constants of prednisolone and methylprednisolone acetates (**IX, X**) with β-CD are the same as those of the corresponding alcohols (**VII, VIII)**. The association constants of acetates with γ-CD are greater than of the corresponding alcohols. On the other hand esterification of betamethasone (**XI**) and dexamethasone (**XII**) with phosphoric acid results in a reduction of K_{a} with β- and γ-CD, by approximately half. The phosphates are soluble in water and ionized in aqueous solutions. This ionized form is less compatible with both CDs than the alcoholic, more hydrophobic form of the steroids.

The association constants for the first four compounds (**I–IV**) were determined previously in methanol-water containing a greater concentration of methanol (45 : 55) at the same temperature [1, 7]. The constants in methanol–water (20 : 80) are about 20 times greater for complexes with β-CD, and about 10 times greater for complexes with γ-CD. A stronger dependence of the association constant with β-CD can be due to the different numbers of complexed methanol molecules in the CD cavity in both cases or to a greater obstruction on the part of methanol molecules to association in the smaller cavity of β-CD. The value of the association constant of methanol with β-CD is 0.32 M^{-1} at 25 °C [14], but to our knowledge this value for γ-CD has not been measured.

There are several reports on the reduction of the association constants of complexes in methanol-water solution with increasing methanol concentration [6, 15, 16]. Similar effects were found in other mixtures of several organic solvents with water: dimethylsulfoxide [17], ethanol [18, 19], formamide [18]. The association constants in pure dimethylsulfoxide were 1–2 orders of magnitude lower than in pure H_2O [20]. These facts support the supposition that competition with solvent molecules is an important contribution to the stability of the complexes. Taraszewska measured polarographically [4] the stability of complexes of chloronitrobenzenes in the mixtures of water and several organic solvents: she found that the addition of these solvents caused a reduction of the association constant as compared to that in pure water but after taking into account the concentration of free cyclodextrin, uncomplexed by the organic solvent molecules, the calculated association constants were the same as measured in pure water. As a matter of fact there are also some reports giving the opposite view that the formation constants of pyrene/CD complexes measured in the presence of various alcohols are enhanced with respect to that measured in the absence of alcohols [21, 22]. Both reports concern the results obtained by fluorescence measurements: the change of fluorescence lifetimes [21] or the vibronic band ratio [22] of pyrene in the presence of CD.

It is interesting that the association constants of estrone (**IV**) with β- and γ-CD are equal within experimental errors, and for estriol the association constant with β-CD is even greater than with γ-CD. For all other compounds the reverse is true, as well as for the same two compounds in methanol–water (45 : 55) [1]. (The data in methanol–water (45 : 55) given in [7] are too large in the case of estriol–β-CD; the lower values determined previously [1] were confirmed later by the Hummel–Dreyer method: unpublished results). The complexes of estriol (**III**) and estrone (**IV**) with β-CD were also weaker than with γ-CD in acetonitrile–water (30 : 70) (unpublished data). For estradiol (**I**), both association constants are the same within experimental error in methanol–water 20 : 80. In the 45 : 55 mixture the value of K_a with γ-CD was twice as large as with β-CD.

4. Conclusions

The present results did not solve the question of which part of a steroid molecule, which is too bulky to enter the β- and γ-cyclodextrin cavities as a whole, participates in the complexation. The data in Table I show that the structures of both peripheries of the steroid molecule skeleton influence the stability of their complexes with β- and γ-CD. One possible explanation of this finding is that the complexed substrate molecules present several molecular orientations.

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