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Report

Beta-Cyclodextrin/Steroid Complexation: Effect of Steroid Structure on Association Equilibria

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Molecular associations of beta-cyclodextrin (beta-CyD) with four steroids (cortisone, hydrocortisone, progesterone, and testosterone) have been studied using phase-solubility and spectroscopic techniques. Phase solubility diagrams could be categorized as B type. The complexes are formed at the stoichiometric ratios of 1:2 (drug:beta-CyD). A mathematical model has been proposed to calculate the apparent stability constants. The results suggest that the inclusion of a steroid molecule into the first beta-CyD cavity is thermodynamically more favorable over the association of 1:1 complex with the second beta-CyD molecule except for cortisone, which exhibits anomalous behavior. A mechanism of complexation has been proposed based on the apparent stability constants and chemical structures of the steroids and beta-CyD. It suggests that complexation is first brought about by inclusion of the five-member cyclopentane ring of the steroid molecule into the first beta-CyD cavity. The 1:1 complex subsequently binds with the second beta-CyD to form the 1:2 complex. The association constants of steroid/beta-CyD complexes are of the following order: progesterone > cortisone > testosterone > hydrocortisone. The order of aqueous solubilities of the complexes is hydrocortisone > cortisone > testosterone > progesterone.

KEY WORDS: beta-cyclodextrin; complexation; steroids; apparent stability constants.

INTRODUCTION

Beta-Cyclodextrin (beta-CyD) is able to form inclusion complexes with a variety of poorly water-soluble compounds, thereby increasing their aqueous solubility and systemic availability (1,2). The interaction between beta-CyD and steroids has been previously investigated (3–6). However, the effects of steroid structures on the complexation equilibria have not yet been clarified. To understand the mechanism of molecular association, one must know the apparent stability constants and complex stoichiometry. Various approaches to estimate these parameters have already been discussed (7–9). In particular, mathematical methods for the analysis of the A-type 1:1 plus 1:2 complex system have been described (10,11). The apparent stability constants associated with the complexation between beta-CyD and steroids were calculated previously without correctly determining the stoichiometric ratio (5,6). In those studies, the apparent stability constants were calculated assuming a stoichiometric ratio of 1:1. However, in our study the stoichiometric ratio has been found to be 1:2 (steroid:beta-CyD), and the apparent stability constant for the second equilibrium K_2 has been determined. Moreover, four steroids (shown in Fig. 1) have been chosen as model compounds to investigate the inclusion mechanism. Both equilibrium constants, K_1 and K_2, have been estimated by utilizing a model in which both equilibria are considered simultaneously.

THEORETICAL

The following equilibria may be used to describe a two-step 1:2 association process where S represents the substrate, i.e., steroid, and L represents the ligand, i.e., beta-CyD:

$$S + L \overset{K_1}{\rightleftarrows} SL \tag{1}$$

$$SL + L \overset{K_2}{\rightleftarrows} SL_2 \tag{2}$$

In the presence of excess solid substrate, Eq. (3) may describe the total substrate concentration in solution as a function of total ligand concentration

$$[S]_t = \frac{K_1[S]_0 + K_1K_2[S]_0[L]}{1 + K_1[S]_0 + 2K_1K_2[S]_0[L]}[L]_t + [S]_0 \tag{3}$$

where

K_1 = $[SL]/[S]_0[L]$
K_2 = $[SL_2]/[SL][L]$
$[S]_0$ = solubility of substrate (steroid)
$[S]_t$ = total drug concentration in solution
$[L]$ = concentration of free ligand (beta-CyD) in solution
$[L]_t$ = total concentration of ligand (beta-CyD) in solution

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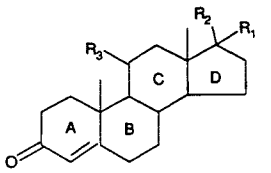

	R_1	R_2	R_3
I	H	$COCH_3$	H
II	H	OH	H
III	OH	$COCH_2OH$	O
IV	OH	$COCH_2OH$	OH

Fig. 1. Steroid structures: I, progesterone; II, testosterone; III, cortisone; and IV, hydrocortisone.

For the sake of simplicity, Eq. (3) may be written as

$$[S]_t = \frac{1 + K_2 \cdot [L]}{1 + 1/(K_1 \cdot [S]_0) + 2K_2 \cdot [L]} [L]_t + [S]_0 \quad (4)$$

When $2K_2 \cdot [L] \ll 1$, then Eq. (2) simplifies to

$$[S]_t = \frac{1}{1 + 1/(K_1 \cdot [S]_0)} [L]_t + [S]_0 \quad (5)$$

Equation (5) may be rewritten as

$$[S]_t = \frac{K_1 \cdot [S]_0}{1 + K_1 \cdot [S]_0} [L]_t + [S]_0 \quad (6)$$

A linear relationship between $[S]_t$ and $[L]_t$ is obtained according to Eq. (6). The slope of an $[S]_t$ versus $[L]_t$ plot according to Eq. (6) can be defined as shown in Eq. (7):

$$\text{slope} = \frac{K_1[S]_0}{1 + K_1[S]_0} \quad (7)$$

Rearrangement of Eq. (7) yields Eq. (8), which is most commonly used for calculating the apparent stability constant of 1:1 complexes, i.e.,

$$K_1 = \frac{\text{slope}}{[S]_0(1 - \text{slope})} \quad (8)$$

However, the stability constant of a 1:2 complexation process is dependent on both K_1 and K_2, since $K_{overall}$ is the product of K_1 and K_2. Thus, it is necessary to determine K_2. Because K_1 and K_2 are equilibrium constants, a third proportionality constant K_3 can be defined as

$$K_3 = K_1/K_2 \quad (9)$$

and therefore

$$K_3 = [SL]^2/[SL_2]\,[S]_0 \quad (10)$$

For a B-type complexation, when a saturation with respect to the complexes is reached, the total drug concentration in solution remains constant as presented in Eq. (11).

$$[S]_t = [S]_0 + [SL]_0 + [SL_2]_0 = \text{constant} \quad (11)$$

where $[S]_0$ is the solubility of the steroid, and $[SL]_0$ is the concentration of 1:1 complex present in the plateau region of the solubility phase diagram. $[SL_2]_0$, the solubility of the 1:2 complex, can be obtained from the descending portion of the solubility phase diagram or by using pure solid complexes. Since $[S]_0$ and $[SL_2]_0$ are measurable, $[SL]_0$ can be estimated using Eq. (11).

The term K_3 can be redefined as

$$K_3 = [SL]_0^2/[S]_0[SL_2]_0 \quad (12)$$

Equation (12) defines the specific conditions existing at the end of the plateau region where the solution is saturated with respect to the free steroid and 1:1 and 1:2 complexes. From Eqs. (8), (10), and (12), Eq. (13) can be obtained, which expresses K_2 in terms of the 1:1 and 1:2 complex concentrations.

$$K_2 = K_1/K_3 = \frac{[SL_2]_0 \cdot \text{slope}}{[SL]_0^2(1 - \text{slope})} \quad (13)$$

Two assumptions are made in the derivation of Eq. (13).

(i) The total concentration $[S]_t$ at the end of the plateau region in the solubility diagram satisfies Eq. (11).

(ii) Since a very low concentration of beta-CyD was used in the initial portion of the solubility phase diagram, the value of the term $2K_2 \cdot L$ is much smaller than 1, an assumption which from the data presented later appears to be true.

MATERIALS AND METHODS

Materials. Beta-CyD, testosterone, cortisone, and hydrocortisone were purchased from Sigma Chemical Company, St. Louis, MO. Progesterone was obtained from Aldrich Chemical Company, Milwaukee, WI. Beta-CyD was recrystallized from deionized double-distilled water, which was used throughout the study.

Phase Solubility Study. These experiments were performed in 10-ml liquid scintillation vials (Research Products International Corp., Mount Prospect, IL) immersed in a thermostated water bath (MGW Lauda, Postfech, West Germany) kept at 30 ± 0.5°C. For each experiment, a steroidal compound in the amount of 0.05 mmol was accurately weighed into a vial to which 5 ml of an aqueous solution containing various concentrations of beta-CyD (0.01 to 25 m*M*) was added. The content in each vial was continually stirred with a small magnetic stirrer for 16 hr. The solid in the equilibrated solution was filtrated off using a 0.4-μm polycarbonated nucleopore membrane (Nucleopore, Pleasanton, CA). Only the last 2 ml of the filtrate was collected, appropriately diluted with water, and finally analyzed at 243 nm on a Beckman–Du7 spectrophotometer (Beckman Instruments, Westbury, NY) equipped with a data handling computer system.

Preparation of the Complexes. The pure beta-CyD complexes of all the steroids were obtained from the descending portion of the solubility phase diagram where no free solid drug exists. The solid residue was dried *in vacuo* at 65°C over phosphorous pentoxide.

Characterization of the Complexes. The stoichiometric ratio of the complexes was calculated by three methods, i.e., (a) phase solubility diagram, (b) fast atom bombardment

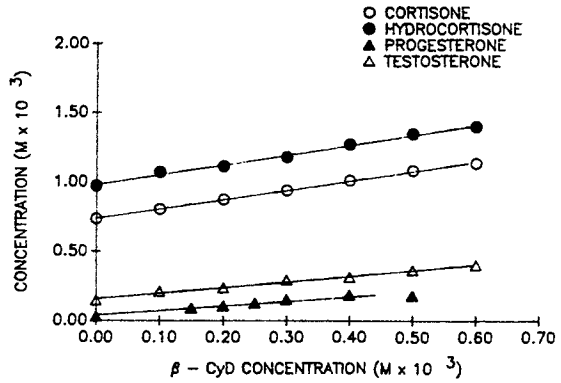

Fig. 2. The initial portions of the phase solubility diagrams of steroid–beta-CyD systems in water at 30°C.

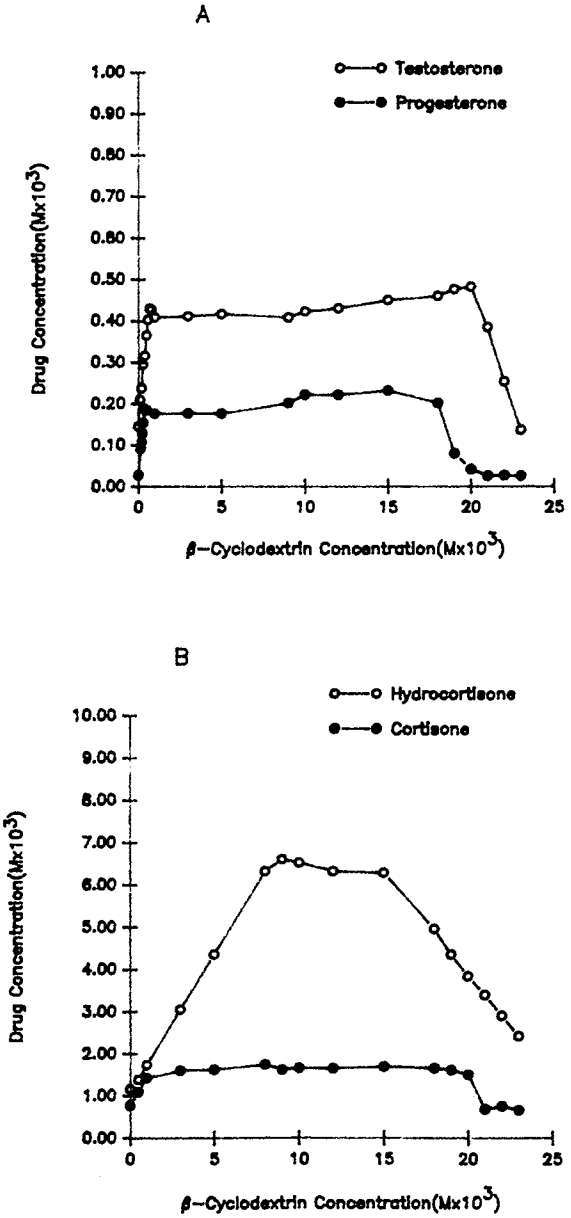

Fig. 3. Phase solubility diagrams of progesterone, testosterone–beta-CyD (A) and cortisone, hydrocortisone–beta-CyD (B) systems in water at 30°C.

mass spectral analysis of pure complexes using a Kratos MS-50 sector mass spectrometer, and finally, (c) chemical analysis of the pure solid complexes.

A scanning rate of 100/sec and an accelerating voltage of 8 kV were applied to obtain the MS spectra. Dithiothreitol (DTT)/dithioerythritol (DTE), or glycerin was used as the sample matrix. The solid residue obtained from 10 m*M* beta-CyD solution as mentioned earlier was analyzed by the same methods as their corresponding complexes prepared by collecting the solid present in the descending portion of the phase solubility diagram. The chemical and physical properties of the complexes were examined with differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) techniques. DSC was performed with Perkin Elmer DSC-4 at a heating rate of 20°C/min, and indium was used as a standard indicator of the melting point. FTIR spectra were obtained from a Nicolet FTIR spectrometer. The KBr pellet method was utilized in the FTIR study.

Determination of Dissociation Constant Using Pure Complex. A known amount (50 mg) of pure solid complex of each steroid was weighed into a vial containing 5 ml water. After the system was equilibrated, appropriate dilutions were made and the drug concentrations were determined by UV analysis. In this case, the solution is saturated and the total drug concentration is presumably contributed by *SL* and SL_2 species, since the drug solubility is much smaller than the total drug concentration. The free beta-CyD concentration in the system is, therefore, equal to the concentration of *SL*. The solubility of the 1:2 complex was determined by adding 25 mg of the 1:2 complex into 5 ml of solutions containing 5 and 10 m*M* beta-CyD in order to suppress any dissociation of the 1:2 complexes. K_2 is calculated according to the definition given previously.

RESULTS AND DISCUSSION

The solubility phase diagrams obtained from equilibrium solubility studies (Figs. 2–3) have shown B-type behavior (7) for the beta-CyD/steroid systems. The initial portion of the solubility phase diagram shows a significant increase in total steroid concentration as a result of complexation. The initial increase in steroid solubility is much greater than the solubility behavior observed in the latter portion of the solubility phase diagram at high beta-CyD concentrations; the solubility approximates that of the pure 1:2 steroid complex when beta-CyD concentration is above 20 m*M*. Analysis of the solid phase from this region by mass spectra and chemical analysis of pure complex indicates that the complexes in the solid state have a stoichiometric ratio of 1:2

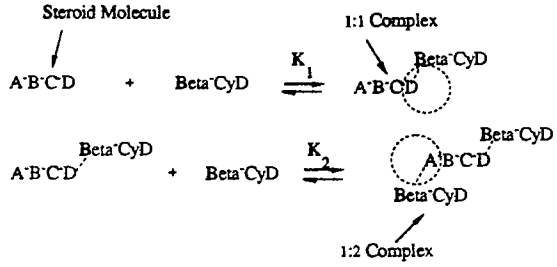

Fig. 4. Schematic of a complexation process between a steroid molecule and two beta-CyD molecules.

Table I. Mass Fragments of the Complexes Obtained from the FAB Mass Spectra

Complex	Mass fragment (*m/e*)	Matrix
Progesterone	1136, 1159, 1440,[a] 1592, 2267, 2289, 2576,[b] 2736	DTT/DTE
Testosterone	1136, 1159, 1286, 1328, 1348, 1389, 1424,[a] 1439, 2559[b]	Glycerin
Cortisone	1136, 1159, 1286, 1441, 1496,[a] 1592, 2267, 2289, 2576, 2631,[b] 2736	DTT/DTE
Hydrocortisone	1136, 1159, 1286, 1441, 1498,[a] 1592, 2289, 2576, 2633,[b] 2736	DTT/DTE

[a] 1:1 complex ion
[b] 1:2 complex ion.

(steroid:beta-CyD). The mass fragments obtained from the FAB mass spectra are listed in Table I. Both 1:1 and 1:2 complex molecular ions were seen in the spectra as noted in Table I.

The apparent stability constants (Table II) were calculated according to the model described previously. It is apparent from the magnitude of K_1 and K_2 that the steroid molecule first forms an inclusion complex with one molecule of beta-CyD, which apparently is a more favored process over the binding of the 1:1 complex with the second molecule of beta-CyD. In the case of cortisone, the data suggest that the association of the 1:1 complex with the second beta-CyD molecule is a favored process over the first association. Such anomalous behavior could be due to the formation of a 1:1 complex which results in the breakdown of the intramolecular H bonding of cortisone formed between the ketone group at the 11 position and the primary hydroxyl group of the side chain at the 17 position. The free ketone group may increase the lipophilicity of the 1:1 complex and may enhance the compound's ability to undergo hydrophobic association with the second beta-CyD. It should be noted that although K_2 is greater than K_1 for cortisone, the calculation of both K values is still valid as the assumption that $K_2 \cdot [L] \ll 1$ still applies. The K_2 values obtained from the dissociation of the pure complex agrees well with the K_2 values obtained according to the proposed model.

In contrast to cortisone, hydrocortisone has a secondary hydroxyl group at the 11 position which is able to form an intramolecular H bond with the ketone group of the side chain at the 17 position. The formation of the 1:1 complex breaks down this H bond and frees the secondary hydroxyl group at the 11 position, resulting in an increase in the chemical polarity and a decrease in the ability of the 1:1 complex to bind with another beta-CyD molecule. This finding may suggest that the 1:1 complex was first formed between the five-member cyclopentane ring of the steroid and a beta-CyD molecule. The second step then involves the inclusion of the free A ring into the second beta-CyD cavity to form the 1:2 complex. A tentative mechanisms of inclusion process has been proposed in Fig. 4 which can be used to explain the association of all four steroids.

DSC diagrams (Fig. 5) illustrate sharp melting behavior for both the drug powders and the physical mixtures but not for pure complexes and beta-CyD. This observation implies that the molecular arrangement of steroids in the solid complexes is different from that in their own crystal habit; indeed, it probably indicates that the steroids are dispersed at a molecular level in the solid complexes.

The functional groups of the steroids involved in the complexation were investigated by FTIR spectrometry. The wavenumber change of the conjugated carbonyl group at the 3 position and the carbonyl groups at the 11 and 20 positions before and after complexation demonstrated orbital energy transfer in the system. For example, the FTIR spectrum (Fig. 6) of the cortisone/beta-CyD complex exhibits a shift in wavenumber of the carbonyl group at the 3 position by about 24 cm^{-1}, whereas there is no change in the spectra of physical mixture. The magnitude of the shift of wavenumber given by the same group on the same position of steroid rings is different for each steroid (Table III). This indicates that complexation between the steroid and beta-CyD in aqueous solution is also dependent on the side chains of the steroid.

In summary, the steroids can form inclusion complexes with beta-CyD at a stoichiometric ratio of 1:2. Such com-

Table II. Mean Apparent Stability Constants of the Complexation Between Beta-CyD and the Steroids

Compound	K_1 (M^{-1})	K_2 (M^{-1})	K_2[a] (M^{-1})	$K_{overall}$ (M^{-2})	$\Delta G°$ (kcal/mol)
Progesterone	24,705 (23,494–29,515)[b]	686 (437–863)	871 (631–1,038)	1.7×10^7	10.02
Testosterone	5,058 (4,904–5,204)	2763 (2,537–2,904)	2,044 (1,833–2,255)	1.4×10^7	9.99
Hydrocortisone	2,683 (1,967–2,931)	140 (33–247)	324 (205–443)	3.7×10^5	7.72
Cortisone	2,632 (2,426–2,864)	5,697 (5,114–6,280)	4,177 (3,872–4,482)	1.5×10^7	10.00

[a] Calculated from the dissociation study.
[b] Numbers in parentheses denote the ranges of calculated equilibrium constant values.

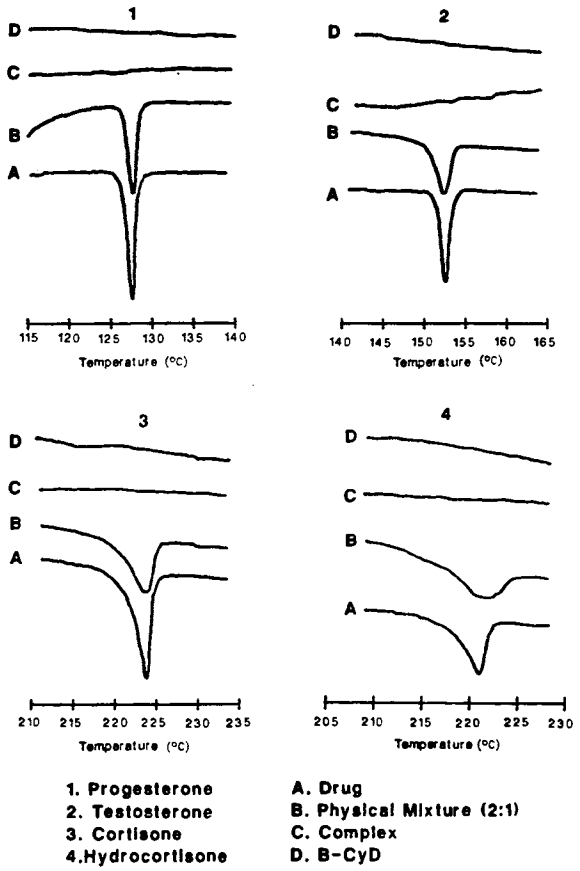

Fig. 5. DSC thermogram of beta-CyD, steroid, steroid–beta-CyD physical mixture, and complexes.

plexation takes place initially between the five-member cyclopentane ring (D ring) of the steroid and the hydrophobic cavity of the beta-CyD molecule. The 1:1 complex then binds with the second beta-CyD to form the 1:2 complex.

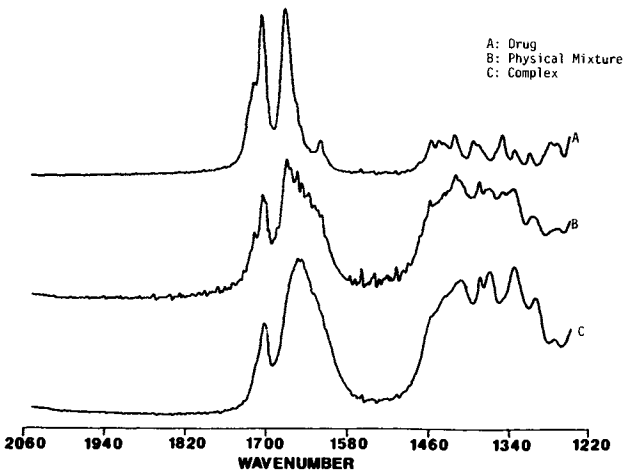

Fig. 6. FTIR spectra obtained from cortisone, cortisone–beta-CyD physical mixture, and cortisone complex by the KBr pellet method.

Table III. Wavenumber Shift of the Carbonyl Groups of Steroids Prior to and After Complexation with Beta-CyD in IR Spectra

Steroid	Wavenumber shift (cm^{-1})[a]		
	C-3	C-11	C-20
Progesterone	7.0 (1651)[b]	—	0 (1702)
Testosterone	8.0 (1656)	—	—
Hydrocortisone	4.0 (1643)	—	4.0 (1708)
Cortisone	24.0 (1667)	No peak (1718)	4.0 (1703)

[a] Wavenumber shift is obtained from the difference between the pure complex and the physical mixture.

[b] The number in parentheses is the initial wavenumber corresponding to the various carbonyl groups in the steroid molecule in the absence of beta-CyD.

This step involves the association of the A ring of steroid already partially inserted into one beta-CyD with the second beta-CyD molecule. As can be observed from the phase solubility diagrams, the 1:1 complexes of all four steroids are more polar than their corresponding 1:2 complexes. This study therefore indicates that the interactions and thermodynamics of steroid complexation with beta-CyD are very much dependent on the steroid structure.

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