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Short Communication

Inclusion complexes of steroid hormones with cyclodextrins studied by the Hummel–Dreyer method using reversed-phase liquid chromatography*

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Introduction

Inclusion complexes between cyclodextrins (CDs) and a number of smaller molecules were studied by various methods, including chromatography [1–5]. Chromatographic investigation of the equilibria between a molecule and its complexing agent, taking place in solutions, usually consists of the measurement of the decrease in the capacity factor of a compound on addition of the complexing agent to the eluent. This method was recently applied for the evaluation of association constants (K_a) of the complexes of steroid hormones with β-CD [4]. Here, another approach, known as the Hummel–Dreyer method was applied for the same purpose using β-CD and γ-CD. This method is based on the following procedure [5, 6]. A column is equilibrated with an eluent carrying a solute molecule (S). A sample of the complexing agent (C) is prepared in the eluent solution and applied to the column. If sufficient complexation occurs and if C and the complex SC move faster than S, the chromatogram will contain the positive peak of SC 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 SC. The height of the horizontal base line 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. This method was devised for gel filtration chromatography and was used with the columns which are suitable for this technique (e.g. LiChrosorb Diol for the study of propranolol binding to human α_1-acid glycoprotein [7]). Here the method is adopted for RP HPLC on an alkyl-bonded silica column (ODS). The aim of this paper is to determine K_a values of complexes of four steroid hormones with β-CD and γ-CD. The results for β-CD complexes were compared with those obtained in the earlier studies under the same conditions (eluent, temperature) [4]. Complexes with γ-CD were not studied previously on account of relatively high price of the reagent, a great deal of which was needed. In the Hummel–Dreyer method the consumption of ligands is minute.

Experimental

Materials

β-CD was purchased from the Sigma Chemical Co., (St Louis, USA). γ-CD was from Merck (Darmstadt, Germany). Water content in CDs was determined by the Karl–Fisscher method. Stock solutions of steroids containing about 0.6 mM of the compounds in the case of estradiol, ethinyloestradiol and estriol, and about 0.3 mM in the case of estrone in methanol–water (45:55, v/v) were prepared. (Concentration of the estrone stock solution was lower because of its lower solubility). The

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stock solutions were diluted tenfold or fivefold (estrone) to produce eluents. Solutions for calibration were obtained in a similar manner but concentrations of steroid in them were twice as large as in the eluents. Solutions for injection containing CDs were obtained by dissolving a weighed amount of CD (about 40 mg) in 5.5 ml H_2O, adding 4.5 ml of methanol, and adjusting with methanol–water (45:55), to 10 ml. Concentration of CD in the solution was about 3 mM. 1 ml of the stock steroid solution (or 2 ml in the case of estrone) were diluted to 10 ml by the CD solution. Dissolution of a weighed amount in the eluent would be more convenient but was impossible because β-CD is not soluble in this concentration in the methanol–water mixture. On the other hand less methanol in the eluent would result in too large elution volumes.

Apparatus

The LC system used was Shimadzu pump (LC-10AS), oven (CTO-10AC), UV detector (SPD-10A), integrator R6A, and Philips refractive index detector PU 4026. The column used was Partisil ODS-1 (25 cm × 4.6 mm internal diameter), mean particle size 10 μm. Volumes of the injected samples were 50 μl. Detection was effected at 280 nm, i.e. the absorption maximum of the eluent. The calibration solution was injected onto the column through a Rheodyne loop injector alternatively with the solution of CD.

Results and Discussion

The recorded elution profiles showing pairs of positive and negative peaks visualized complexation of the four steroids with β- and γ-CD. As an example, the elution profile of estriol with γ-CD is shown in Fig. 1. The first positive peak is attributed to the estriol-γ-CD complex (S-CD). Behind it absorption continues to be constant and then the negative peak appears, which corresponds to estriol defficiency. Association constants were calculated according to

$$K_a = \frac{Q_{S-CD}}{[S]_o\,(Q_{CD} - Q_{S-CD})}, \qquad (1)$$

where Q_{S-CD} — total amount of the complex, Q_{CD} — total amount of CD applied, $[S]_o$ — initial concentration of steroid in the eluent.

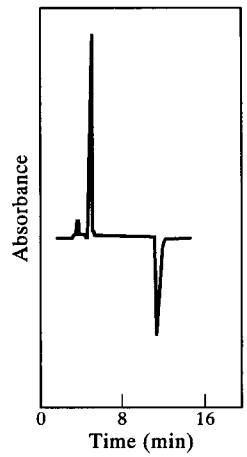

Figure 1
LC elution profile obtained after injection of 50 μl of γ-cyclodextrin solution (about 2.7 mM) in methanol–water, (45:55, v/v), containing 0.06 mM of estriol, onto RP 18 column equilibrated with the eluent containing estriol of the same concentration in the same solvent.

Equation 1 is valid if the complex stoichiometry is 1:1; this ratio being found for the complexes of the four steroids with β-CD in [4]. Here the method of Hummel and Dreyer was used to determine the complex stoichiometry in a straightforward way. If 1:1 complex is formed, then, according to equation (1), the application to the column of a given amount, Q_{CD}, of CD should produce twice as large 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 dependence of K_a on Q_{CD} is different

$$K_a = \frac{Q_{S-CD_2}}{[S]_o\,(Q_{CD} - 2\,Q_{S-CD_2})^2}. \qquad (2)$$

The amount of the complex formed after application of one-half of CD, $Q_{CD}/2$, can be calculated for the individual values of Q_{CD} and Q_{S-CD_2}. (The ratio of the complex amounts in the two cases is near to 4.) One can conclude the stoichiometry of the complex formed from the comparison of the experimental ratio with the calculated one.

The above procedure was used for estriol. For the complex with β-CD the measured ratio was 1.95 (assuming 1:2 stoichiometry, the calculated value being 3.7). For the complex with γ-CD the measurement gives 2.03 whereas for the 1:2 complex the calculated value is 3.8. This confirms the formation of the 1:1 complex.

Table 1
Association constants (M^{-1}) and their standard deviations of four steroid complexes with β- and γ-CD (methanol–water (45:55, v/v)), at 35°C

Steroid	Method of Hummel–Dreyer		Method using capacity factors [4]
	β-CD	γ-CD	β-CD
Estradiol	342 ± 43 n = 10	738 ± 94 n = 6	399 ± 18
Ethinylo-estradiol	379 ± 15 n = 10	995 ± 96 n = 4	269 ± 11
Estrone	278 ± 58 n = 10	356 ± 65 n = 10	224 ± 8
Estriol	532 ± 90 n = 10	498 ± 33 n = 8	223 ± 3

For the evaluation of $Q_{S\cdot CD}$, the positive peaks were used, as suggested in [8]. The determined values of K_a are shown in Table 1. It can be seen that with the exception of estriol, the complexes with γ-CD are stronger that with β-CD. K_a of complexes with β-CD are compared with the corresponding values from [4]. It can be seen from the table that K_a of estradiol and estrone determined by the Hummel–Dreyer method and the change of capacity factor method [4] are similar and within experimental errors; the values for ethinyloestradiol and estriol are higher with the Hummel–Dreyer approach. The reason for these differences is not clear although in the capacity factor method some assumptions have to be made and verification of the degree of retention of the ligand and its complexes must be made [9]. On the other hand the Hummel–Dreyer method used in this study is insensitive to the degree of retention of the complex and complexing agent by the stationary phase. The sole condition is that the complex can be washed out of the column earlier than the free ligand. To ascertain which method produced better results it would be worth using a third, independent method for the same purpose.

The association constant of α-CD with estriol was also evaluated and found to be very small (14 M^{-1}), and so the association constants with α-CD were not determined for the other compounds.

In the method of Hummel–Dreyer the time of the complex elution is seen on the chromatogram as the retention time of the positive peak. This gives a capacity factor for the complexes with β-CD of about 1.3, and about 0.15 with γ-CD, for all the compounds investigated. These capacity factors are the same as those of the free CDs measured on the same column and at the same temperature, but without a steroid compound in the eluent. Chromatograms of free CDs were recorded with the aid of the refractive index detector. γ-CD and its complexes were only slightly retained by the column. β-CD and its complexes were retained to a higher degree as is expected from their more hydrophobic character [10]. Nevertheless, it should be remembered that the conclusions about the retention of complexes refer to the conditions where the column is saturated with the steroid which is complexed by injection of CD.

Conclusions

It has been shown that the Hummel–Dreyer method can be used for the determination of the association constants of complexes using octadecylsilane (ODS) columns. This method can be also used for the investigation of complex stoichiometry in solutions which may differ from the stoichiometry of a precipitated complex, also some differences in stoichiometry may occur due to the solvents used. The utilization of columns of various types makes the Hummel–Dreyer method flexible and worthy of attention in the investigation of such effects.

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