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Improvement of Chemical Instability of Digitoxin in Aqueous Solution by Complexation with β-Cyclodextrin Derivatives

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Inclusion complexation of digitoxin with β-cyclodextrin (β-CyD) derivatives, such as 2,6-di-*O*-methyl-β-CyD (DM-β-CyD), 2,3,6-tri-*O*-methyl-β-CyD (TM-β-CyD), hydroxypropyl-β-CyD (HP-β-CyD, substitution degree 4.3) and hydroxyethyl-β-CyD (HE-β-CyD, substitution degree 4.0) was studied by the solubility method. The apparent 1 : 1 stability constant (K_c) of the complexes in water at 25 °C was in the order of DM-β-CyD ($84000\,M^{-1}$) > HP-β-CyD ($18000\,M^{-1}$) ≈ HE-β-CyD ($17000\,M^{-1}$) ≈ β-CyD ($17000\,M^{-1}$) > TM-β-CyD ($5600\,M^{-1}$). From the high-performance liquid chromatographic tracing of each of the four components (digitoxin, bisdigitoxoside, monodigitoxoside, digitoxigenin) in the reaction mixtures, the individual hydrolysis rate constants (k_1—k_6) were determined by the non-linear least-squares method. β-CyDs suppressed the hydrolysis rates of digitoxin species in an acidic medium (pH 1.2), particularly the appearance of digitoxigenin, the final hydrolysis product, and the inhibitory effect was generally in the order of DM-β-CyD > β-CyD ≈ HP-β-CyD ≈ HE-β-CyD > TM-β-CyD. By analyzing the concentration dependency of the hydrolysis rate, DM-β-CyD was found to decelerate the appearance rate of digitoxigenin more than 2400 times.

Keywords——digitoxin; acid hydrolysis; bisdigitoxoside; monodigitoxoside; digitoxigenin; β-CyD derivative; complexation; stability constant; stabilization; concentration dependency

The potent cardiac glycosides such as digoxin and digitoxin are known to be susceptible to hydrolysis in an acidic medium, which may result in decreased therapeutic efficiency as well as oral bioavailability.[1-3] In previous papers,[4-6] we reported that the chemical instability and oral bioavailability of digoxin were improved by α-, β- and γ-cyclodextrin (α-, β- and γ-CyD) complexations. In this series of studies, the effects of various β-CyD derivatives, such as 2,6-di-*O*-methyl-β-CyD (DM-β-CyD), 2,3,6-tri-*O*-methyl-β-CyD (TM-β-CyD), hydroxypropyl-β-CyD (HP-β-CyD) and hydroxyethyl-β-CyD (HE-β-CyD), on the acid hydrolysis of digitoxin were kinetically investigated in the hope of achieving greater hydrolytic stability than could be obtained with the parent β-CyD.

Experimental

Materials——β-CyD and DM-β-CyD were donated by Nippon Shokuhin Kako Co., Ltd. and Toshin Chemical Co., Ltd., respectively, and used after recrystallization from water. TM-β-CyD was prepared according to the method reported by Hakomori.[7] HP- and HE-β-CyDs were supplied by Toshin Chemical Co. and Wako Chemical Co., and their average degrees of substitution were confirmed to be 4.3 and 4.6, respectively, by secondary ion mass spectrometry (SIMS) as reported previously.[8] Digitoxin, bisdigitoxoside, monodigitoxoside and digitoxigenin were purchased from Böehringer Mannheim GmbH (FRG). All other materials and solvents were of analytical reagent grade and deionized double-distilled water was used throughout the study.

Solubility Studies——Solubility measurements were carried out according to Higuchi and Connors.[9] For example, excess amounts (8 mg) of digitoxin were weighed into 10-ml vials and to each vial was added an aqueous solution (3 ml) containing various concentrations of β-CyDs. The vials were sealed with glass stoppers and parafilm (American Can Co., U.S.A.), and shaken in a constant-temperature room at 25±0.2 °C. After equilibrium was

reached (about 10 d), an aliquot was centrifuged and pipetted through a cotton filter (the adsorption of digitoxin on the cotton filter was negligible). The filtered aliquots were analyzed by spectrophotometry at 220 nm after adequate dilution with water. Under these experimental conditions, no degradation of digitoxin was observed. Apparent 1 : 1 stability constants (K_c) of the complexes were calculated from the initial straight-line portion of the phase solubility diagrams and expressed as means of at least three determinations with a maximal standard deviation of 1000.

$$K_c = \frac{\text{slope}}{\text{intercept } (1 - \text{slope})} \tag{1}$$

Kinetic Studies——The acid hydrolysis of digitoxin species in the presence and absence of CyDs was carried out in HCl–KCl buffer (pH 1.2, $\mu = 0.2$) at 37 °C. The reaction was initiated by addition of a stock solution of the parent compound in methanol to the buffer solution in 20-ml vials placed in a thermostated (±0.1 °C) water bath. The final concentrations of substrates and methanol were 2.5×10^{-5} M and 5% (v/v), respectively. A 0.5 ml sample solution was withdrawn at timed intervals, added to 2 ml of 0.2 M phosphate buffer (pH 7.5), and subjected to high-performance liquid chromatography (HPLC) for simultaneous determination of the parent compound and its hydrolytic products. The pH of the sample solution was ascertained to be identical before and after the reaction.

HPLC Analysis——The chromatograph was operated in a constant-temperature room at 25±0.2 °C. The flow rate was 1.0 ml/min and the elute was monitored spectrophotometrically at 220 nm. The separation utilized a LiChrosorb RP-18 column (5 μm in 4 mm × 25 cm, Merck, FRG), with acetonitrile–methanol–water (30 : 35 : 35) as the mobile phase. Components were quantitated by measuring peak heights and comparing the height with those of known amounts of internal standard, digoxin. A typical chromatogram is shown in Fig. 1, where digitoxin and its hydrolysis products were well separated.

Determination of Rate Constants——The acid hydrolysis of digitoxin is known to involve a complex combination of parallel and consecutive reactions, as shown in Chart 1. The disappearance rate of digitoxin (D) and appearance rates of hydrolysis products [bisdigitoxoside (B), monodigitoxoside (M) and digitoxigenin (G)] can be described by pseudo-first-order equations, *i.e.*, Eqs. 2—5.

$$-\frac{dD}{dt} = (k_1 + k_2 + k_3)D \tag{2}$$

$$\frac{dB}{dt} = k_1 D - (k_4 + k_5)B \tag{3}$$

$$\frac{dM}{dt} = k_2 D + k_4 B - k_6 M \tag{4}$$

$$\frac{dG}{dt} = k_3 D + k_5 B + k_6 M \tag{5}$$

The individual rate constants in Chart 1 were determined by a non-linear least-squares method combined with the Runge–Kutta–Gill method, using the MULTI program.[10]

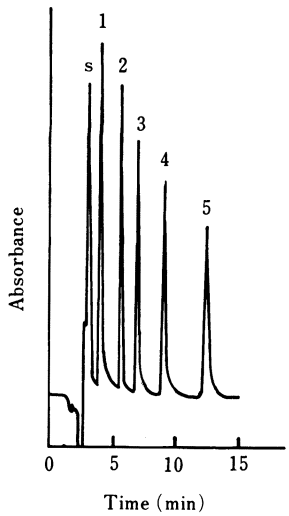

Fig. 1. Liquid Chromatogram of Digitoxin and Its Hydrolysis Products

1, digoxin (internal standard); 2, digitoxigenin; 3, monodigitoxoside; 4, bisdigitoxoside; 5, digitoxin; s, solvent.

Operating conditions are described in the text.

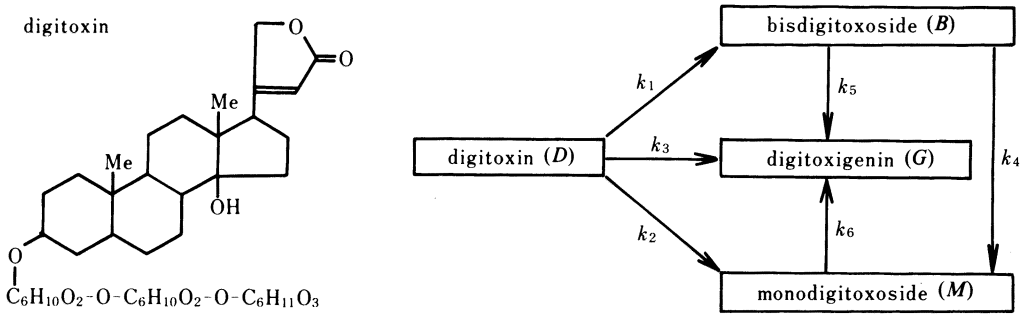

Chart 1

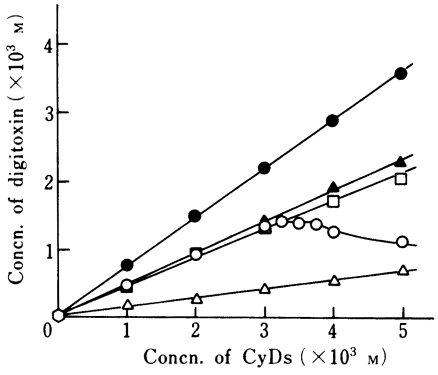

Fig. 2. Phase Solubility Diagrams of Digitoxin–CyD Systems in Water at 25 °C

○, β-CyD; ●, DM-β-CyD; △, TM-β-CyD; ▲, HP-β-CyD; □, HE-β-CyD.

Results and Discussion

Inclusion Complexation of Digitoxin with β-CyDs

The inclusion complexation of digitoxin with β-CyD derivatives was studied by the solubility method. Figure 2 shows the phase solubility diagrams obtained for digitoxin–CyD systems in water at 25 °C. The solubility plot for β-CyD showed a B_s-type solubility curve showing precipitation of the solid complex in the higher β-CyD concentration regions. On the other hand, those for β-CyD derivatives showed A_L-type curves, *i.e.*, the solubility of digitoxin (2.5×10^{-5} M) increased in a linear fashion as a function of CyD concentration. The apparent 1 : 1 stability constant (K_c) of complexes was calculated from the initial linear portion of the solubility diagrams according to Eq. 1, and was in the order of DM-β-CyD ($84000\ M^{-1}$) > HP-β-CyD ($18000\ M^{-1}$) ≈ HE-β-CyD ($17000\ M^{-1}$) ≈ β-CyD ($17000\ M^{-1}$) > TM-β-CyD ($5600\ M^{-1}$). Similar strong bindings to DM-β-CyD were observed for steroidal hormones, as reported previously.[11]

Effects of β-CyDs on the Hydrolysis Rate of Digitoxin

Figure 3 shows typical examples of variation in the composition of digitoxin hydrolysis products in the presence and absence of β-CyDs in acidic medium (pH 1.2) at 37 °C. Each hydrolysis product in the hydrolyzate was expressed as the mole fraction of all digitoxin species present. It is apparent from Fig. 3 that β-CyDs retarded the hydrolysis rate of digitoxin, and the appearance of digitoxigenin, a final product of the hydrolysis, was almost completely inhibited. To gain insight into the decelerative mechanism of β-CyDs, therefore, the individual hydrolysis rate constants (k_1—k_6) in Chart 1 were determined by analyzing the time-conversion profiles of Fig. 3 according to Eqs. 2—5, and the results are summarized in Table I. The theoretically calculated concentrations of each component, using the rate

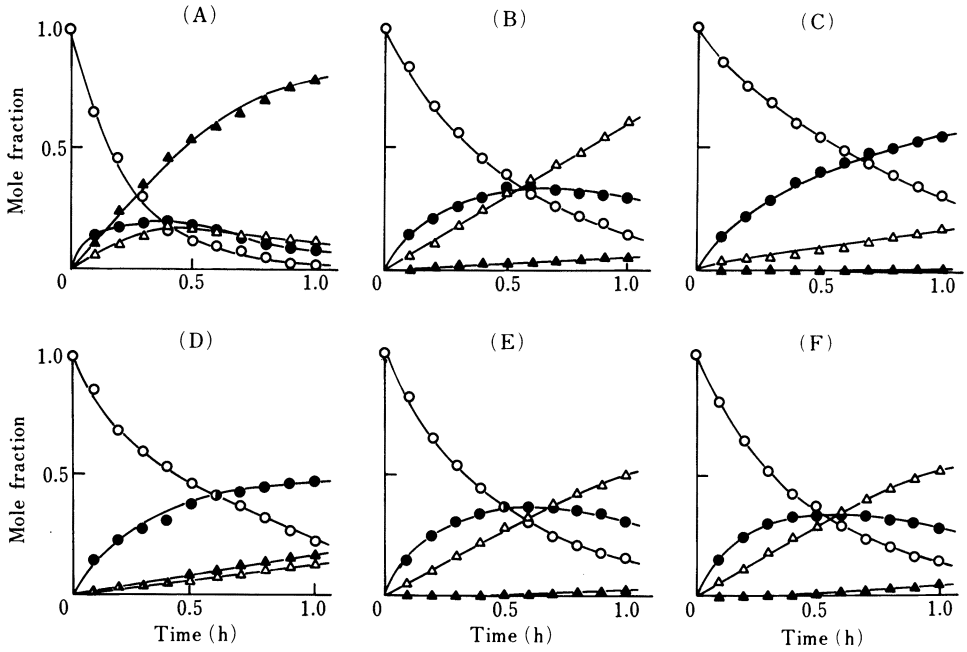

Fig. 3. Time-Conversion Profiles for Digitoxin (2.5×10^{-5} M) Hydrolysis in the Presence and Absence of β-CyDs (2×10^{-3} M) at pH 1.2 and 37 °C

(A) Without CyD. (B) With β-CyD. (C) With DM-β-CyD. (D) With TM-β-CyD. (E) With HP-β-CyD. (F) With HE-β-CyD.
○, digitoxin; ●, bisdigitoxoside; △, monodigitoxoside; ▲, digitoxigenin.
The solid line was calculated by using the rate constants given in the text.

TABLE I. Rate Constants (h^{-1})[a] for Hydrolysis of Digitoxin in the Absence and Presence of β-CyDs (2×10^{-3} M) at pH 1.2 and 37 °C

Rate constant	Without CyD	With β-CyD	With DM-β-CyD	With TM-β-CyD	With HP-β-CyD	With HE-β-CyD
k_1	1.55	1.50	1.09	1.21	1.37	1.44
k_2	0.576	0.545	0.115	0.140	0.430	0.494
k_3	1.40	0.035	0.025	0.159	0.061	0.046
k_4	2.75	1.24	0.221	0.315	0.955	1.00
k_5	0.120	0.074	<0.001	0.136	<0.001	0.090
k_6	3.08	0.020	<0.001	0.845	0.041	<0.001

a) The averages of at least three determinations, which coincide with each other within ±5%.

constants in Table I, were in fair agreement with the experimentally determined values, as shown in Fig. 3. It is apparent that digitoxin was hydrolyzed *via* six routes (Chart 1), although two routes (k_2 and k_5) were less important than other routes, possibly due to the steric restrictions imposed at the glycosidic bonds. By the addition of β-CyDs, the relative weightings of the hydrolysis pathways were changed; *i.e.*, the conversions of digitoxin to digitoxigenin (k_3, k_5 and k_6) were selectively suppressed, and the inhibitory effect of β-CyDs was generally in the order of DM-β-CyD > HP-β-CyD ≈ HE-β-CyD ≈ β−CyD > TM-β-CyD. In the case of TM-β-CyD, the conversion to monodigitoxoside (k_2 and k_4) was also suppressed, suggesting that the inclusion mode of TM-β-CyD may be different from those of other β-CyDs.

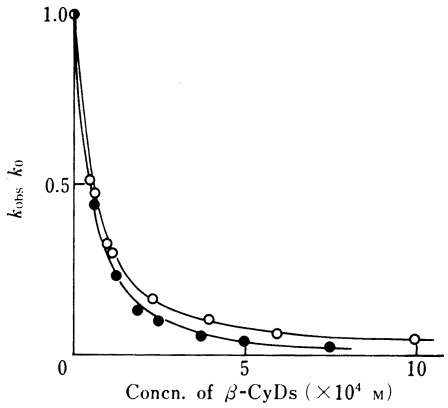

Fig. 4. Observed Rate Constants (k_{obs}) for the Hydrolysis of Monodigitoxoside as a Function of Concentration of β-CyDs at pH 1.2 and 37 °C

○, β-CyD; ●, DM-β-CyD.

TABLE II. Rate Constants (k_c, h^{-1})[a] and Stability Constants (K_c, M^{-1}) of Monodigitoxoside–CyD Complexes at pH 1.2 and 37 °C

System	k_0	k_c	k_0/k_c	K_c
Monodigitoxoside	2.42	—	—	—
β-CyD complex	—	0.023	100	24900
DM-β-CyD complex	—	<0.001	>2400	117000

a) The averages of at least three determinations, which coincide with each other within ±5%.

Effects of β-CyD and DM-β-CyD on the Hydrolysis of Monodigitoxoside to Digitoxigenin

Prevention of the appearance of digitoxigenin might be clinically important since the cardioactivity of digitoxigenin is several times weaker than that of digitoxin, but other glycosides possess approximately the same activity.[12] As mentioned above, β-CyDs inhibited the hydrolysis of digitoxin, particularly the conversion step of digitoxin species to digitoxigenin, and the inhibitory effect was the largest for DM-β-CyD among β-CyD derivatives. Therefore, the effect of DM-β-CyD concentration on the hydrolysis rate of monodigitoxoside to digitoxigenin was investigated in detail and compared with that of parent β-CyD. As shown in Fig. 4, the hydrolysis rate hyperbolically decreased with increasing β-CyD concentration. These concentration dependencies of the apparent rate constants (k_{obs}) were quantitatively treated by Eq. 6 to obtain the apparent K_c value and rate constants (k_c) of the complexes, assuming a 1 : 1 complexation scheme.[13]

$$\frac{(CyD)_t}{k_0-k_{obs}}=\frac{1}{k_0-k_c}(CyD)_t+\frac{1}{K_c(k_0-k_c)} \quad (6)$$

where k_0 and $(CyD)_t$ are the rate constant in the absence of CyDs and the total concentration of CyDs, respectively. The plots according to Eq. 6 were linear with a correlation coefficient greater than 0.999. The results on k_0, k_0/k_c and K_c are summarized in Table II. The kinetically determined K_c values were similar in magnitude to those obtained from the solubility diagrams as described above, although the chemical structure of the guest molecules and the experimental conditions were all different. It is worth noting that the hydrolysis of the glycosidic linkage between digitoxigenin and monodigitoxose was decelerated by factors of 100 and more than 2400 by inclusion complexations with β- and DM-β-CyDs, respectively. Such a large decelerative effect of DM-β-CyD has rarely been observed in other CyD systems, as far as we know.

In conclusion, the hydrolytic lability of digitoxin in acidic aqueous solution was

significantly improved by β-CyD derivatives, particularly DM-β-CyD. It is also important to note that the stabilizing effects of HP- and HE-β-CyDs were almost the same as that of the parent β-CyD. Thus, the present data may be particularly useful from the viewpoints of stabilization of digitoxin as well as oral digitoxin therapy, because the irritative effects of HP- and HE-β-CyDs on the gastrointestinal tract are weaker than that of the parent β-CyD.[8)]

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