Inclusion Complexation of *p*-Hydroxybenzoic Acid Esters with 2-Hydroxypropyl-β-cyclodextrins. On Changes in Solubility and Antimicrobial Activity

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To obtain a transparent and effective solution of *p*-hydroxybenzoic acid esters (parabens), the use of 2-hydroxypropyl-β-cyclodextrins (2-HP-β-CyDs) as solubilizers with different degrees of substitution (D.S.) was surveyed. 2-HP-β-CyDs significantly increased the aqueous solubility of four kinds of parabens (methyl < ethyl < propyl < butyl esters), where the solubilizing ability decreased with an increase in the D.S. of the 2-hydroxypropyl group in β-CyD. The antimicrobial activity of the parabens tended to decrease by complexation with 2-HP-β-CyDs. However, the activity could be maintained by lengthening the alkyl chain of the parabens. ^{1}H- and ^{13}C-nuclear magnetic resonance and circular dichroism spectroscopic studies suggest that the hydrophobic alkyl moiety of butyl paraben is preferably included in the cavity, and the phenol group extrudes from the cavity. The present results suggest that a suitable combination of 2-HP-β-CyDs and hydrophobic, longer alkyl parabens is useful for the preservation of liquid formulations.

Keywords *p*-hydroxybenzoic acid ester; 2-hydroxypropyl-β-cyclodextrin; inclusion complex; solubilization; antimicrobial activity

Chemically modified cyclodextrins (CyDs) have been utilized for the improvement of pharmaceutical properties of drug molecules, *e.g.*, solubility, chemical stability or bioavailability.[1–3] So-called hydroxyalkylated CyDs are amorphous mixtures of chemically related components with different degrees of substitution (D.S.).[4–6] Their multi-component character prevents any crystallization, thus hydroxyalkylated CyDs have high solubility (>50%) in water. Among the currently available hydroxyalkylated CyDs, 2-hydroxypropyl-β-CyDs (2-HP-β-CyDs) deserve special attention since their physicochemical properties and biological features have been well demonstrated.[7,8]

p-Hydroxybenzoic acid esters (parabens) are widely used as typical preservatives for medicines, cosmetics and foods. In general, the prolongation of the alkyl chain of parabens increases not only their antiseptic action, but also their clinical safety.[9] However, practical use of parabens with longer alkyl chains has been limited because of their low solubility in water. In the present study, the inclusion complexation of parabens with 2-HP-β-CyD with different D.S. values was studied in detail by nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopies. Furthermore, the solubilization of parabens by 2-HP-β-CyDs and the resulting antimicrobial activity-changes were investigated in order to ultimately obtain a transparent and effective paraben solution.

Experimental

Materials 2-HP-β-CyDs with five different D.S. values (2.5, 3.0, 4.3, 7.4 and 12.0) of the 2-hydroxypropyl group ($-CH_2CH(OH)CH_3$) were prepared by Nihon Shokuhin Kako Ltd. (Shizuoka, Japan). The D.S. was expressed as the average number of 2-hydroxypropyl groups per β-CyD molecule, which was determined as reported previously.[7,10] Four kinds of parabens (Wako Pure Chemical Industries, Ltd., Osaka, Japan), *i.e.*, methyl, ethyl, propyl and butyl esters of *p*-hydroxybenzoic acid, were used as supplied. All other chemicals were of analytical reagent grade.

Solubility Measurements A constant but excess amount of paraben was added to an aqueous solution containing a given concentration of 2-HP-β-CyD. These were mixed by a magnetic stirrer at 25 °C, and after equilibrium was attained (about 12 h) the mixture was filtered. Paraben in the filtrate was assayed by high performance liquid chromatography (HPLC), under the following conditions, using a JASCO 860-CO HPLC apparatus (Tokyo, Japan). Column, CAPCELL-PAK C18 SG 4.6 × 250 mm (Shiseido, Yokohama, Japan); detection, UV at 256 nm; mobile phase, water : methanol = 3 : 7 (v/v); amount of injection, 10 μl; flow rate, 1.0 ml/min.

Determination of Stability Constants The phase solubility diagrams were prepared by the method of Higuchi and Connors,[11] and an apparent 1 : 1 stability constant (K', M^{-1}) was calculated from the slope and intercept values of the initial straight line portion of the solubility diagram, according to the following equation:

$$K' = \text{slope}/\{\text{intercept} \cdot (1 - \text{slope})\} \quad (1)$$

Measurements of Antimicrobial Activities The effect of 2-HP-β-CyD (D.S. = 3.0) on the antibacterial activities of parabens was determined by evaluating the mortality rate of a microorganism which had been seeded/vaccinated with the bacterial strain 13, *Candida albicans* ACCT-10231, at 10^4 cell/ml in an air-tight glass container in which the sample was placed. To determine the minimum inhibitory concentration (*MIC*), paraben which had been diluted at various stages in potato dextrose agar culture medium was adjusted to the respective concentration range in the culture media. After seeding/vaccinating the culture medium with a fixed quantity of the *Candida albicans* ACCT10231, the *MIC* value was determined by evaluating the state of growth of the bacteria. The concentration of 2-HP-β-CyD was adjusted with water to make various concentrations (*ca.* 7.0% (w/v)), whereas paraben was diluted with water to prepare a concentration range of 50—2500 μg/ml. 2-HP-β-CyDs were confirmed to have no antimicrobial activity under the experimental conditions. *MIC* values were determined from an average (±30 ppm, S.D.) of three measurements.

NMR and CD Measurements ^{1}H-NMR spectra were taken at 25°C on a JEOL JNM FX-270 spectrometer (Tokyo, Japan) operating at 270.17 MHz with a sweep width of 2700 Hz. ^{13}C-NMR spectra were taken on a JEOL GX-400 operating at 100.53 MHz with a sweep width of 15000 Hz at 50°C, because of the low solubility of butyl paraben in water. The concentrations of methyl and butyl parabens were 2.0 and 1.0 mM for ^{1}H-NMR spectra and 10 and 5 mM for ^{13}C-NMR spectra, respectively, in deuterium oxide (D_2O). ^{1}H- and ^{13}C-chemical shifts were measured, using sodium 2,2-dimethyl-2-silapentane-5-sulfonate and dioxane as external references, with an accuracy of ±0.0011 and ±0.014 ppm,

respectively. CD and UV spectra were recorded with a JASCO J-50A recording polarimeter and a Hitachi U-3200 spectrophotometer, respectively, and all measurements were carried out in a pH 7.0 sodium phosphate buffer ($\mu=0.2$) at 25 °C.

Results and Discussion

Solubilization Figure 1 shows a typical example of the phase solubility diagram of paraben-2-HP-β-CyD (D.S. = 4.3) systems in water. The solubility of each paraben linearly increased as a function of 2-HP-β-CyD concentration, showing features of an A_L type diagram.[11] Similar results were obtained for other 2-HP-β-CyDs with different D.S. values. This solubility behavior was in sharp contrast to the case of parent β-CyD where crystalline complexes precipitated at higher host concentrations (>0.01 M), showing a typical B_s type solubility curve.[12] The solubilization of parabens by 2-HP-β-CyD was attained in the order of butyl > propyl > ethyl > methyl parabens, with an increase in the hydrophobic nature of the guest molecules. The superior solubilizing ability of 2-HP-β-CyD prevented the precipitation of paraben crystals during storage. For example, a 0.2% (w/v) solution of butyl paraben containing 2-HP-β-CyD (2.0% (w/v)) in ethanol–glycerin–water (1 : 1 : 8 (v/v)) gave no crystals and the solution remained transparent even after 30 d at 5 °C. On the other hand, 0.1% (w/v) of butyl paraben alone in the solvent gave fine crystals after storage for 1 d at 5—50 °C.

The apparent stability constants (K') were determined from the intercept and slope of the straight lines of the solubility diagrams, assuming a 1 : 1 complex formation.[11] As shown in Table I, the K' value increased with an increase in the alkyl chain of the paraben, while it tended to decrease with an increase in the D.S. of 2-HP-β-CyDs, probably due to the steric hindrance of the host molecules as reported previously.[6,7] These results indicate that the hydrophobic nature of a guest molecule, together with the spatial relationship between the host and guest molecules, largely contributes to the inclusion complexation of parabens with 2-HP-β-CyDs in water.

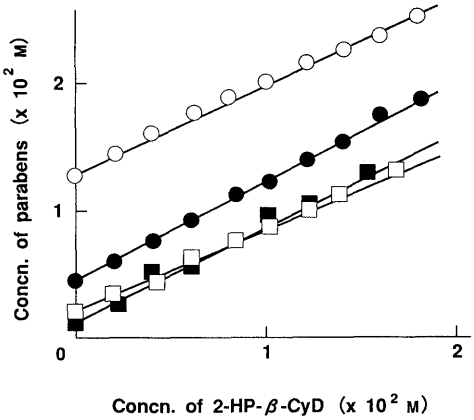

Fig. 1. Phase Solubility Diagrams of Methyl (○), Ethyl (●), Propyl (□) and Butyl (■) Paraben-2-HP-β-CyD (D.S. = 4.3) Systems in Water at 25 °C

TABLE I. Apparent Stability Constants (K', M^{-1})[a] of Paraben Complexes with 2-HP-β-CyD with Different D.S. in Water at 25 °C

D.S.	Methyl	Ethyl	Propyl	Butyl
3.0	180	840	1440	4310
4.3	150	770	1400	3600
7.0	140	740	1300	3550
12.0	90	520	1270	2240

a) Average of the value for duplicate measurements, which coincide with each other within ±2%.

Confirmation of Inclusion Complexation by NMR and CD Spectroscopies The UV absorption maximum (255 nm) of methyl and butyl parabens shifted to a slightly longer wavelength (about 2 nm), with a concomitant decrease in the molar absorption coefficient, in the presence of 2-HP-β-CyDs. In the CD spectra, the optical activity of the parabens was induced with positive sign at 253 nm by the addition of 2-HP-β-CyD (D.S. = 3.0), and the CD intensity increased with an increasing concentration of the host, as shown in Fig. 2. The concentration dependence of CD intensity was analyzed according to the method of Benesi–Hildebrand[13] assuming a 1 : 1 complexation, and the K' value and molar ellipticity (θ) of the complexes were as follows: 300 M^{-1} and 9.4×10^3 degree·M^{-1} and 3500 M^{-1} and 5.8×10^3 degree·M^{-1}, respectively, for the methyl and butyl paraben complexes. The plots of the Benesi–Hildebrand equation were linear (correlation coefficient > 0.99), supporting the 1 : 1 complexation. The (θ) value of

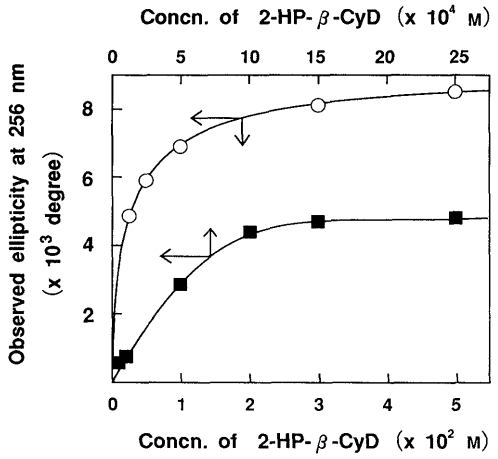

Fig. 2. Changes in Observed Ellipticity at 256 nm of Methyl (○) and Butyl (■) Parabens as a Function of 2-HP-β-CyD (D.S. = 3.0) Concentration in Sodium Phosphate Buffer (pH 7.0, $\mu=0.2$) at 25°C

The concentration of parabens was 5.0×10^{-5} M.

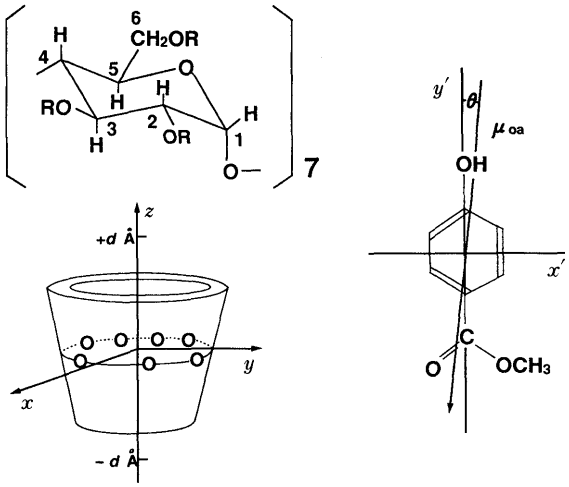

Fig. 3. Coordinate Systems for β-CyD (Left) and Paraben (Right)

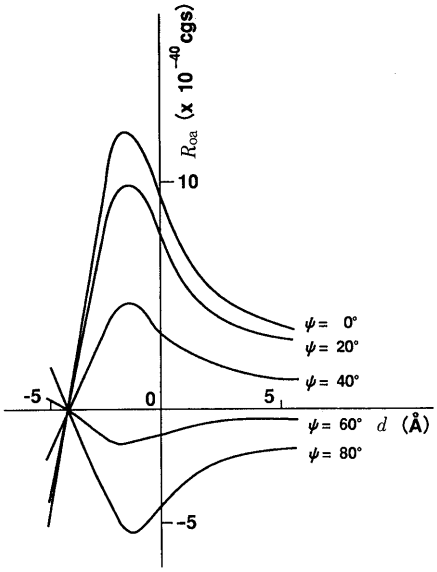

Fig 4. Dependence of Calculated Rotational Strength (R_{oa}) on the Distance (d) and the Angle (ψ)

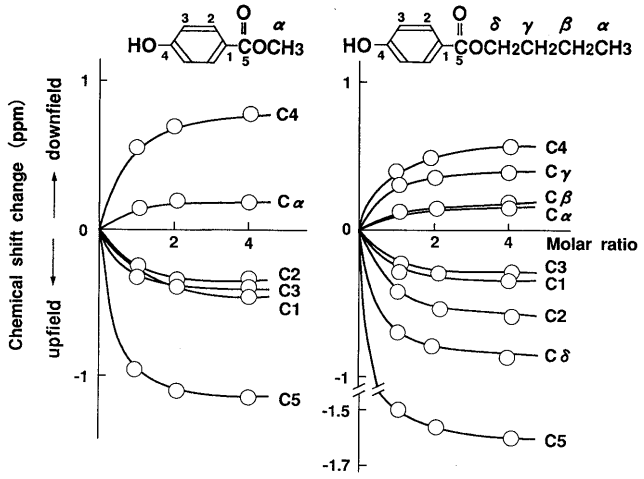

Fig. 5. Changes in ^{13}C-Chemical Shifts of Methyl and Butyl Parabens as a Function of 2-HP-β-CyD (D.S. = 3.0) Concentration in D_2O at 50 °C

the methyl paraben complex was larger than that of the butyl paraben complex, in spite of the smaller K' value of the former complex, suggesting that the chromophore of methyl paraben is more deeply inserted into the cavity, in comparison with the butyl derivative.

It has been reported that the electronic transition of the included guest molecule with a transition dipole moment (μ_{oa}), parallel to the z-axis (see Fig. 3) of the CyD cavity gives a positive CD, while with a transition dipole moment perpendicular to the z-axis, it gives a negative CD.[14,15] In order to gain insight into the inclusion structure, the theoretical rotational strengths (R_{oa}) of parabens in the CyD cavity were calculated by the method of Kirkwood and Tinoco,[16] and compared with the experimental values. Figure 4 shows the calculated R_{oa} of the paraben-β-CyD complexes as a function of the distance (d) between the center of the μ_{oa} and the host, and as a function of the angle (ϕ) between the μ_{oa} and the z-axis. In the calculation, the coordinates of β-CyD with a seven-fold symmetry axis were used because 2-HP-β-CyDs are a multicomponent mixture. All the C6–O6 and O–H bonds of the host were neglected because of their flexibility, and all C–H bonds were neglected because of their isotropic polarizability. As is apparent from Fig. 4, the largest positive CD was given when the center of the μ_{oa} vector of paraben was located at $d = -1.5$ Å and $\phi = 0°$, and the CD intensity decreased when the vector shifted above or below the -1.5 Å plane. Furthermore, the sign of the CD was reversed when the μ_{oa} inclined from the z-axis of the cavity ($\phi > 50°$, minimum at $d = -1.5$ Å and $\phi = 90°$). The experimental R values of the methyl and butyl paraben complexes were detemined to be 6.77×10^{40} and 4.01×10^{40} cgs, respectively, by analyzing the UV and CD spectral data of the complexes according to the following equation[17]:

$$R = 0.696 \times 10^{-42} \pi^{1/2} [\theta]_{max} \Delta / \lambda_{max} \quad (2)$$

where $[\theta]_{max}$ is the maximum value of the molar ellipticity, Δ is the half-band width at $1/e$ of maximum ellipticity and λ_{max} is the wavelength at the absorption maximum. These results suggest that the center of the μ_{oa} vector of parabens should be located within the range of d = about -3—1 Å and -3.5—4 Å for the methyl and butyl paraben complexes, respectively, and that the vactor slightly inclines from the z-axis where the experimental R value is in agreement with the theoretical R_{oa} value. The self consistent field (SCF) linear combination of atomic orbital (LCAO) molecular orbital calculation using the PPP-Cl method[18] suggested that parabens have a transition moment with a large oscillation strength ($f = 0.574$) at about 250 nm, and the moment is almost parallel ($\theta = 6.5°$) to the long molecular axis of paraben, as shown in Fig. 3. These results suggest that the parabens are included in the cavity in the axial mode, and the phenol of methyl paraben is located in the center of the cavity while that of butyl paraben shifts to a wider rim consisting of secondary hydroxyl groups.

Figure 5 shows the displacement of ^{13}C-NMR chemical shifts for methyl and butyl parabens on the addition of 2-HP-β-CyD. For both guest molecules, the *para* carbon (C4) of the benzene and the alkyl carbons, except for the Cδ carbon, showed downfield shifts, while the *ipso* (C1), *ortho* (C2), *meta* (C3) and carbonyl (C5) carbons shifted in the opposite direction. The upfield shift of the C1 carbon and the downfield shift of the C4 carbon indicate that the parabens are included in the 2-HP-β-CyD cavity when the alkyl groups are inserted first, and the p-hydroxyl group is located around the wider secondary hydroxyl rim, as reported by a quantum calculation of solvent effects on ^{13}C shielding.[19] The ^{13}C-chemical shifts of *ortho* and *meta* carbons of substituted benzenes are known to be subject to hydrophobic[19] and steric[20] shieldings, because of close contact with the inner protons of a CyD cavity. For butyl paraben, the upfield shift of the *ortho* carbon (C2) was larger than that of the *meta* carbon (C3), but methyl paraben showed a reversed shift change. This difference became more apparent when the ^{1}H-NMR chemical shifts of the parabens were compared. Figure 6 shows the displacement of ^{1}H-chemical shifts for *ortho* (H2) and *meta* (H3) benzene protons of the guests following the addition of 2-HP-β-CyD. Both protons shifted upfield when the concentration of the host increased. For methyl paraben, the shift of the *meta* proton was larger than that of the *ortho* proton, but for butyl paraben, this was reversed. These results suggest that the phenol moiety of methyl paraben is more deeply inserted

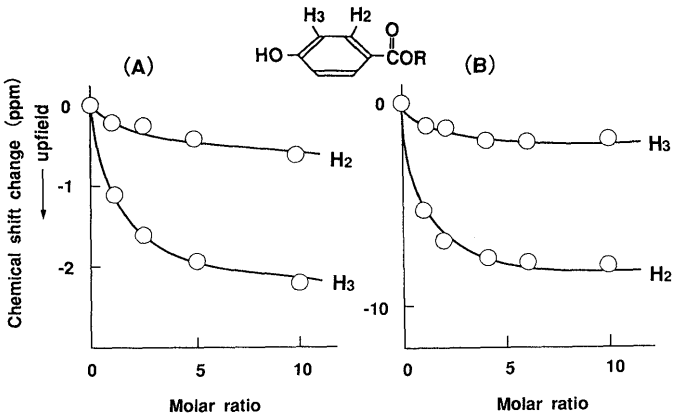

Fig. 6. Changes in *ortho* and *meta* ^{1}H-Chemical Shifts of Methyl (A) and Butyl (B) Parabens as a Function of 2-HP-β-CyD (D.S. = 3.0) Concentration in D_2O at 25 °C

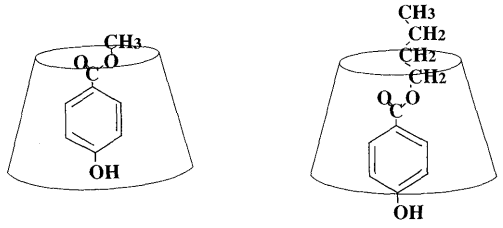

Fig. 7. Proposed Inclusion Structures of Methyl (Left) and Butyl (Right) Paraben-2-HP-β-CyD Complexes in Solution

in the cavity, and that both the *meta* and *ortho* protons and carbons are in close contact with the inner protons of the CyD. On the other hand, only the *ortho* carbons and protons of butyl paraben may be located in the vicinity of the inner protons, *i.e.*, the CyD moves to the hydrophobic alkyl chain of butyl paraben and the phenolic hydroxyl group of the guest protrudes from the wider rim of the cavity, as shown in Fig. 7. The K' values of the 1:1 complexes were determined by analyzing the host concentration dependence of the ^{1}H-chemical shift displacement, according to the method of Hanna *et al.*,[21] and were found to be 270 and 2750 M^{-1} for the methyl and butyl paraben complexes with 2-HP-β-CyD, respectively. These K' values were in good agreement with those determined by the solubility method and the CD spectroscopy described above.

Antimicrobial Activities The effect of 2-HP-β-CyDs on the antibacterial activity of parabens was surveyed, using *Candida albicans* ATCC10231. Figure 8 shows the *MIC* values of each paraben in the presence of various concentrations of 2-HP-β-CyD (D.S. = 3.0). The *MIC* value increased with an increasing concentration of 2-HP-β-CyD. The attenuation of antimicrobial activity may be attributable mainly to the decrease in concentration of parabens in free form. For example, the increasing ratios of the *MIC* value at 2.0% (w/v) 2-HP-β-CyD were 2.0, 2.4, 3.6 and 9.4-fold for methyl, ethyl, propyl and butyl parabens, where the fraction of free parabens were estimated to be 43, 15, 7.5 and 2.3%, respectively, by using the K' values. Of course, it was difficult to calculate accurate fractions of free parabens, because the experimental conditions such as solvency and temperature are different between the

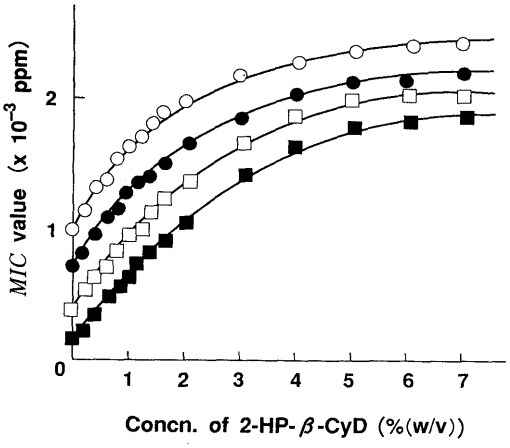

Fig. 8. *MIC* Values of Methyl (○), Ethyl (●), Propyl (□) and Butyl (■) Parabens for *Candida Albicans versus* Concentration of 2-HP-β-CyD (D.S. = 3.0)

MIC values were determined from an average (±30 ppm, S.D.) of three measurements.

solubility method and the antimicrobial activity measurement, and furthermore, competitive inclusion may occur in the culture media. It is of interest that the *MIC* values were saturated at a higher concentration of the host, whereas the antimicrobial activity of parabens was maintained even at such increased host concentrations, although the activity was weak. The *MIC* value at 7.0% (w/v) 2-HP-β-CyD decreased in the same order as that without CyDs (methyl > ethyl > propyl > butyl parabens). Because 2-HP-β-CyD was confirmed to have no antimicrobial activity under these experimental conditions, the saturated *MIC* values may be regarded as those of the complexes; the fractions of free parabens at 7.0% (w/v) 2-HP-β-CyD were 12, 2.8, 1.6 and 0.53% for methyl, ethyl, propyl and butyl parabens, respectively. From practical and safety points of view, the use of butyl paraben/2.0% (w/v) 2-HP-β-CyD may be recommended because of the nearly 14-fold increase in the solubility of butyl paraben while antimicrobial activity was maintained, corresponding to that of methyl paraben.

In conclusion, the low aqueous solubility of parabens, particularly those having long alkyl chains, was significantly improved by inclusion complexation with 2-HP-β-CyDs. The higher solubilization of 2-HP-β-CyD against longer alkyl parabens, compared with short alkyl parabens, may be ascribed to the preferable inclusion of the hydrophobic alkyl chain. The antimicrobial activity of parabens was attenuated by the complexation, but was maintained to some extent in the complexes. The extrusion of the active site, the phenol group, may be at least partly responsible for the maintenance of the antimicrobial activity of the longer alkyl paraben. Longer alkyl parabens such as propyl and butyl parabens are difficult to use practically because of their poor solubility in water. This problem can be solved by the use of 2-HP-β-CyD, which increases the aqueous solubility of the hydrophobic parabens, reducing their turbidity while maintaining their antimicrobial activities at a functional level. Therefore, the data obtained here suggest that 2-HP-β-CyDs have a significant advantage over surface active agents with respect to generating high aqueous solubility while maintaining a lack of toxicity[6,7] in liquid preparations which can be used in medicines and cosmetics.

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