Some Properties and the Inclusion Behavior of Three Positional Isomers of $6^1,6^n$-Di-*O*-α-D-glucosyl-cyclomaltoheptaoses (β-Cyclodextrins)

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Three positional isomers of $6^1,6^n$-di-*O*-α-D-glucosyl-cyclomaltoheptaose [1,*n*-$(G)_2$-βCDs; $n=2$—4] which existed in the digests with glucoamylase of the products from cyclomaltoheptaose (β-cyclodextrin, βCD) and maltose with *Klebsiella pneumoniae* pullulanase, were purified by HPLC. The solubilities of two isomers of those doubly branched βCDs, 1,2- and 1,3-$(G)_2$-βCDs, in water were much higher than those of parent non-branched βCD and mono-branched βCD, 6-*O*-α-D-glucosyl-βCD (G-βCD), while the solubility of another isomer, 1,4-$(G)_2$-βCD, was significantly lower than these two isomers, though it was higher than that of βCD. On the other hand, the solubilities of 1,2- and 1,3-isomers in 10, 30, and 50% (v/v) aqueous methanol at 25 °C were independent of methanol concentrations and their solubilities were the same as those in water at 25 °C. However, that of 1,4-isomer increased with increasing methanol concentrations. The hemolytic activities of 1,*n*-$(G)_2$-βCDs on human erythrocytes in isotonic solution were lower than those of G-βCD and βCD, and became weaker in the order of 1,4- > 1,2- > 1,3-isomers. The complex-forming abilities of 1,*n*-$(G)_2$-βCDs for digitoxin, digoxin, fluorometholone, flurbiprofen, hydrocortisone acetate, and norfloxacin were about the same as those of βCD and G-βCD, whereas reserpine was more difficult to include within 1,*n*-$(G)_2$-βCDs than βCD and G-βCD. Nevertheless, the solubilities of those guest compounds were much more enhanced by 1,*n*-$(G)_2$-βCDs and G-βCD than by βCD.

Key words doubly branched β cyclodextrin; positional isomer; inclusion complex; solubility method; hemolytic activity; solubilization

A great number of branched cyclomaltooligosaccharides (cyclodextrins, CDs) have been prepared for new applications different from those of the conventional non-branched CDs such as cyclomaltohexaose (αCD), cyclomaltoheptaose (βCD), and cyclomaltooctaose (γCD).[1-6] In general, when the saccharide side chains are introduced to CDs by enzymatic reaction, their main products are mono-branched CDs, and the multi branched CDs are also produced as minor components. Typical mono-branched CDs of 6-*O*-α-D-glucosyl-, 6-*O*-α-maltosyl-, and 6-*O*-α-maltotriosyl-CDs were purified, and their some properties and inclusion behavior were already reported.[7-11] However, we have not seen detailed reports relating those of multi branched CDs. Because they are minor components, and also contain plural positional isomers, their purification is very difficult. We found that small amounts of doubly branched CDs, $6^1,6^n$-di-*O*-α-D-glucosyl-βCDs [1,*n*-$(G)_2$-βCDs; $n=2$—4] (Fig. 1), existed in a mixture of glucosyl-βCDs prepared by glucoamylolysis of products from βCD and maltose through the reverse action of *Klebsiella pneumoniae* pullulanase [EC 3.2.1.41].[6]

In this study, three positional isomers of 1,*n*-$(G)_2$-βCDs were isolated by HPLC, and their solubilities in water and in various concentrations of methanol in water, hemolytic activities on human erythrocytes, and inclusion behavior for poorly water-soluble compounds were compared both with each other and with those of the parent non-branched βCD and a mono-branched βCD, 6-*O*-α-D-glucosyl-βCD (G-βCD).

Experimental

Materials A mixture of glucosyl-βCDs was donated kindly by Bio Research Corporation of Yokohama (Yokohama, Japan). βCD was used after recrystallization from water. All the other compounds used were obtained from commercial sources. Deionized and doubly distilled water was used throughout this experiment. Reagent-grade organic solvents used for HPLC were freshly distilled and filtered through a 0.45 μm membrane filter.

General Method A UVIDEC 610C double beam spectrophotometer (Jasco, Tokyo, Japan) was used for the determination of absorbances. HPLC was performed with a PU-980 pump (Jasco), a Rheodyne 7125 injector, a UV-970 variable-wavelength ultraviolet spectrophotometer (Jasco), and a Shodex RI-71 monitor (Showa Denko, Tokyo). HPLC analyses at constant temperature were carried out with an SSC 3510C column oven (Senshu Scientific, Tokyo). The columns employed were a YMC-Pack ODS AQ-323 (250 × 10 mm i.d.) (YMC, Kyoto) for the purification of 1,*n*-$(G)_2$-βCDs and G-βCD, and a TSK gel ODS 4PW (150 × 4.6 mm i.d.) (TOSOH, Tokyo) for analyses of the guest compounds of inclusion complexes.

Solubilities of 1,*n*-$(G)_2$-βCDs The solvent (water or various concentrations of methanol aq. soln.) was carefully added in portions of 0.01—0.1 ml to a glass vessel containing 500 mg of the dried βCDs, and the volume of solvent was measured which was required for complete dissolution of the βCDs within 30 min at 25 ± 1, 40 ± 1, and 55 ± 1 °C, respectively, by vigorous shaking for 30 s periods at 5 min intervals.

Determination of Hemolytic Activity A 0.2% (v/v) human erythrocyte suspension (1 ml) in 0.1 M isotonic phosphate buffer (pH 7.4, PBS) was added to 1 ml of PBS containing various concentrations of βCDs. The mixture was incubated at 37 °C for 30 min and centrifuged at 1300 × *g* for 10 min. Percent hemolysis was expressed in terms of the ratio of the

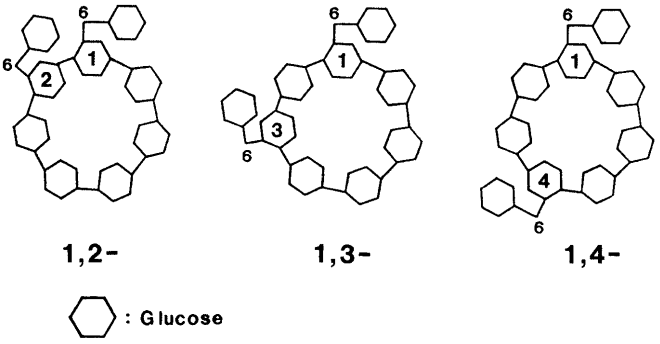

Fig. 1. Schematic Structures of Three Positional Isomers of 1,*n*-$(G)_2$-βCDs

1,2- = 1,2-$(G)_2$-βCD; 1,3- = 1,3-$(G)_2$-βCD; 1,4- = 1,4-$(G)_2$-βCD.

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Table 1. Analytical Conditions of Guest Compound by HPLC

Guest compound	Eluent	Wavelength (nm)	t_R (min)
Hydrocortizone acetate	$CH_3OH:H_2O=50:50$	242	6.8
Fluorometholone	$CH_3OH:H_2O=48:52$	240	6.8
Digitoxin	$CH_3OH:H_2O=60:40$	220	6.4
Digoxin	$CH_3CN:H_2O=26:74$	220	6.3
Flurbiprofen	$CH_3OH:H_3PO_4$ [a] $=68:32$	245	6.6
Norfloxacin	$CH_3OH:H_3PO_4$ [a] $=13:87$	271	6.8
Reserpine	$CH_3OH:H_3PO_4$ [a] $=43:57$	268	6.0

Column, TSK gel ODS-4PW (150 × 4.6 mm i.d.); flow rate, 0.8 ml/min; column temperature, 30 °C. *a*) 0.01% (v/v) H_3PO_4 in water was used.

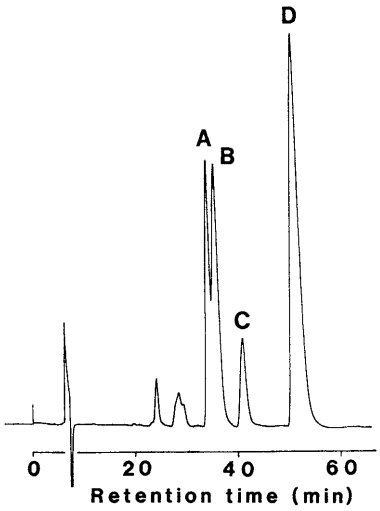

Fig.2. Chromatogram of a Mixture of Glucosyl-βCDs

A, 1,3-$(G)_2$-βCD; B, 1,4-$(G)_2$-βCD; C, 1,2-$(G)_2$-βCD; D, G-βCD. Conditions: column, YMC-Pack ODS AQ-323 (250 × 10 mm i.d.); eluent, CH_3OH–H_2O (7:93); flow rate, 2 ml/min; column temperature, 30 °C.

absorbance at 541 nm of hemoglobin released from erythrocytes with βCDs to the absorbance after the complete hemolysis of erythrocytes in water.

Inclusion Behavior of 1,*n*-$(G)_2$-βCDs Complex-forming abilities of 1,*n*-$(G)_2$-βCDs for poorly water-soluble compounds were estimated according to the solubility method.[13] Excess amounts of the guest compounds were added to the solutions containing various concentrations of βCDs and were shaken at 30 °C. Non-branched βCD has low solubility in water, so that the suspensions of βCD were prepared at over saturated concentration. After equilibrium was attained, an aliquot was pipetted through a 0.2 μm membrane filter, and amounts of the guest compounds in βCD solutions were measured by HPLC. HPLC conditions for analyses of the guest compounds are shown in Table 1.

Results and Discussion

Isolation of Three Positional Isomers of 1,*n*-$(G)_2$-βCDs and G-βCD Figure 2 shows the chromatogram of a mixture of glucosyl-βCDs. It was proven earlier that peaks A, B, and C corresponded to 1,3-, 1,4- and 1,2-$(G)_2$-βCDs (doubly branched βCDs), respectively, and main peak D corresponded to G-βCD (mono-branched βCD).[6] The ratio of doubly branched βCDs and mono-branched βCD in a mixture of glucosyl-βCDs was 1:2, and that of 1,2-, 1,3-, and 1,4-isomers in 1,*n*-$(G)_2$-βCDs was 1:2:2.5. G-βCD and 1,2- and 1,3-isomers were purified by HPLC on an octadecyl silyl silica (ODS) column and 7—10% (v/v) methanol system. 1,4-Isomer which was easily crystallized from water was purified with a combination of HPLC and recrystallization. This characteristic of 1,4-isomer served advantageously in purification of 1,*n*-$(G)_2$-βCDs.

Table 2. Solubility of βCDs in Water at Various Temperatures

βCD	Solubility (mmol/ml × 10^2)		
	25 °C	40 °C	55 °C
1,2-	86	98	114
1,3-	86	98	114
1,4-	2.9	4.3	11
Mix	86	98	114
G-βCD	77	77	133
βCD	1.6	3.1	4.4

Abbreviations of βCDs: 1,2- = 1,2-$(G)_2$-βCD, 1,3- = 1,3-$(G)_2$-βCD, 1,4 = 1,4-$(G)_2$-βCD, Mix = mixture of 1,*n*-$(G)_2$-βCDs (the ratio of 1,2-, 1,3-, and 1,4- isomers are 1:2:2.5). The data is the average of three experiments.

Table 3. Solubility of βCDs in Various Concentrations of Methanol Aqueous Solutions at 25 °C

βCD	Solubility (mmol/ml × 10^2)		
	10%	30%	50%
1,2-	86	86	86
1,3-	86	86	86
1,4-	5.7	21	69
Mix	86	86	86
G-βCD	92	86	77
βCD	1.0	0.5	0.3

Abbreviations are the same as in Table 2. The data is the average of three experiments.

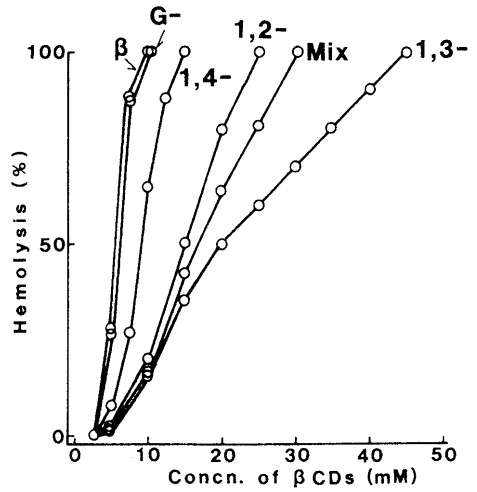

Fig. 3. Hemolytic Effects of βCDs on Human Erythrocytes in 0.1 M Isotonic Phosphate Buffer (pH 7.4)

Abbreviations of compounds are the same as in Table 2.

Solubility Table 2 summarizes the solubilities of 1,2-, 1,3-, and 1,4-isomers of 1,*n*-$(G)_2$-βCDs and that of the mixture of 1,*n*-$(G)_2$-βCDs (Mix) which consisted of three isomers in the ratio of 1:2:2.5, in water at 25, 40, and 55 °C together with data of G-βCD and parent non-branched βCD. The solubilities of 1,2- and 1,3-isomers, and Mix were the same, they dissolved about 54, 32, and 26 times the non-branched βCD at 25, 40, and 55 °C, respectively, and were more soluble than G-βCD at 25

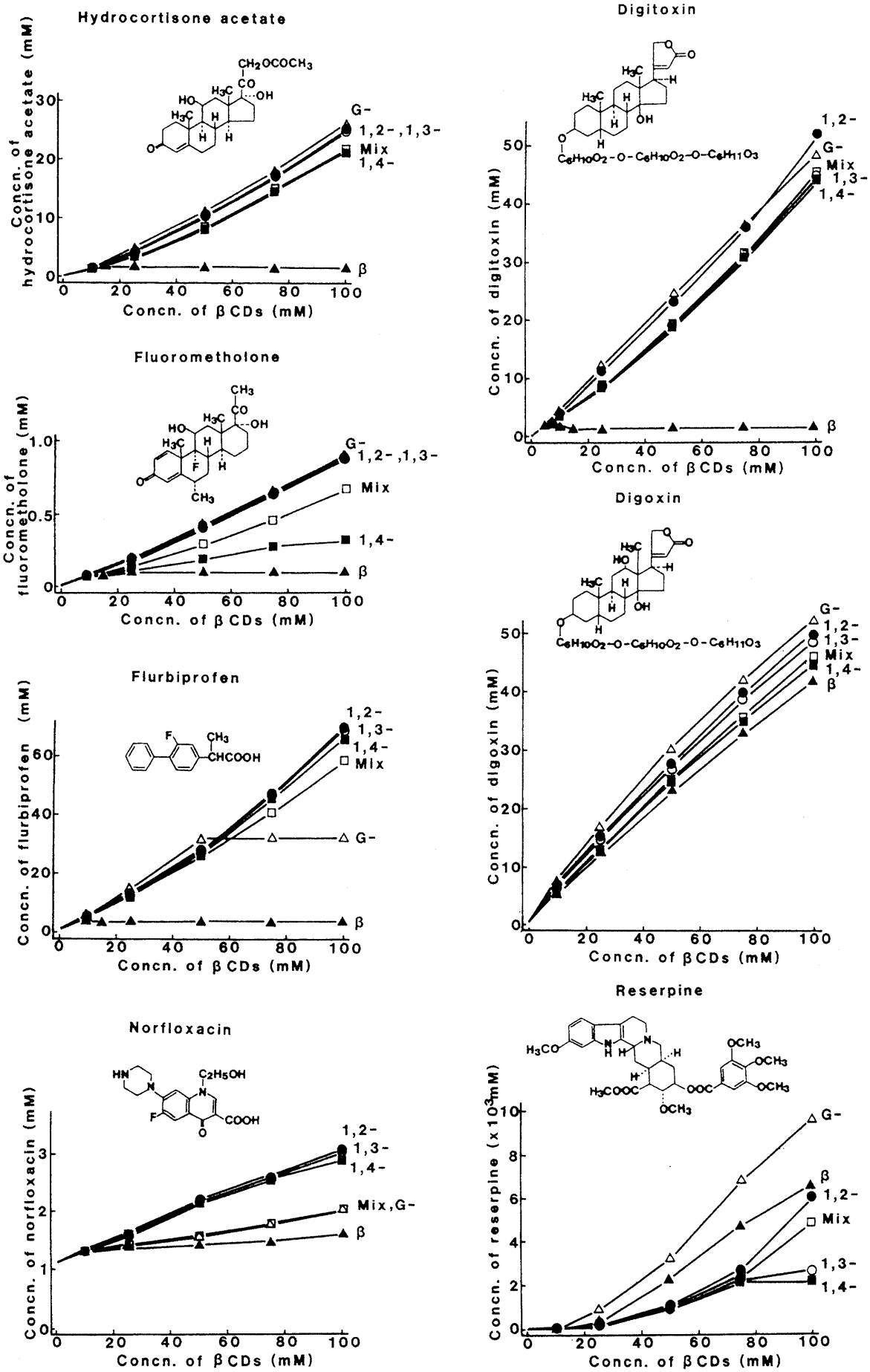

Fig. 4. Phase Solubility Diagrams of Various Guest Compounds with Branched βCDs and βCD in Water at 30 °C

●, 1,2-$(G)_2$-βCD; ○, 1,3-$(G)_2$-βCD; ■, 1,4-$(G)_2$-βCD; □, mixture of 1,*n*-$(G)_2$-βCDs (the ratio of 1,2-, 1,3-, and 1,4- isomers are 1:2:2.5); △, G-βCD; ▲, βCD.

and 40 °C. Although the solubility of 1,4-isomer was higher than that of βCD, it was significantly lower than those of the other two isomers. This is likely to be related to the property of 1,4-isomer which is easily crystallized from water. The solubilities of 1,*n*-$(G)_2$-βCDs in 10, 30, and 50% (v/v) methanol aqueous solutions were examined at 25 °C (Table 3). The solubilities of βCD and G-βCD were decreased with increasing methanol concentrations, while those of 1,2- and 1,3- isomers, and Mix were independent of methanol concentrations, and their solubilities were the same as those in water at 25 °C. Interestingly, only 1,4-isomer had higher solubility in methanol aqueous solutions than in water; moreover, its solubility became greater with increasing methanol concentration.

Hemolytic Activity Figure 3 shows the hemolytic effect of 1,*n*-$(G)_2$-βCDs on human erythrocytes in isotonic solution compared with those of G-βCD and βCD. The hemolytic activities of three positional isomers (1,*n*-$(G)_2$-βCDs) were lower than those of βCD and G-βCD, and became weaker in the order of 1,4- > 1,2- > 1,3-isomers, and that of Mix was situated between the 1,2- and 1,3-isomers. The hemolytic activities of non-branched CDs are known to increase in the order $\gamma < \alpha < \beta$CDs[12]; this order is contrary to that of the solubilities of CDs in water. Accordingly, it is likely that the strongest hemolytic activity of 1,4-isomer among the three isomers is due to its low solubility in water. In spite of the 1,2- and 1,3-isomers having similar solubilities, their hemolytic activities were different. This may be attributed to the different magnitude of interaction between the two isomers and the principal constituents of erythrocyte membrane such as cholesterol and phospholipid, and it may be dependent on the substitution positions of two glucosyl residues on the βCD ring.

Inclusion Behavior The complex-forming abilities of 1,*n*-$(G)_2$-βCDs for several poorly water-soluble (slightly soluble or insoluble) compounds in water were studied by the solubility method and were compared with those of parent βCD and G-βCD. Figure 4 shows the phase solubility diagrams obtained for the various guest compounds with βCDs in water at 30 °C. Table 4 shows the values of apparent stability constant (K), estimated from Eq. 1 based on the assumption that a 1 : 1 complex was

$$K = \frac{\text{slope}}{\text{intercept} \times (1 - \text{slope})} \quad (1)$$

initially formed and calculated from the initial rising portion of the solubility diagrams.[13] No extreme difference could be found among the complexation abilities of the three positional isomers of 1,*n*-$(G)_2$-βCDs, non-branched βCD, and G-βCD, since they have the same size CD cavity. These results are consistent with the concept that the important factor in inclusion complex formation is size compatibility between a guest molecule and the CD cavity.

The solubilities of guest compounds except reserpine were much more enhanced by complex formation with 1,*n*-$(G)_2$-βCDs and G-βCD than with βCD, and most of their solubility isotherms in 1,*n*-$(G)_2$-βCDs and G-βCD solutions were classified as A type, which the solubilities of guest compounds increased in proportion to CD concentration. The very low water-solubility of reserpine was improved with CDs, in the order of G-βCD > βCD > 1,2-isomer > Mix > 1,3-isomer > 1,4-isomer, and B type of solubility isotherm was observed in the solution of 1,4-isomer. It was suggested that two glucosyl residues of 1,*n*-$(G)_2$-βCDs hindered inclusion of the extremely bulky structure of reserpine into the βCD cavity. Also, in the case of fluorometron, 1,4-isomer showed the B type of solubility curve. These two B type solubility isotherms observed in the solution of 1,4-isomer are unusual phenomena in the formation of inclusion complexes with branched CDs. The formation of insoluble complexes with 1,4-isomer may be due to the low solubility of this substance itself in water.

Table 4. Apparent Stability Constants (M^{-1}) of Slightly Soluble Compounds–βCDs Complexes Determined by the Solubility Method in Water at 30 °C

Guest compound	Solubility in H_2O (mM)	Host compounds					
		1,2-	1,3-	1,4-	Mix	G-βCD	βCD
Hydrocortizone acetate	0.03	4700	4700	4200	4200	5000	5000
Fluoromethorone	0.03	280	280	200	240	280	280
Flurbiprofen	0.3	4000	4000	3700	3000	4500	4500
Digitoxin	0.02	37000	34000	34000	34000	38000	37000
Digoxin	0.04	41000	37000	30000	30000	41000	30000
Norfloxacin	1.2	12	12	10	8	8	3

Abbreviations are the same as in Table 2. The data is the average of three experiments. The stability constants of reserpine–βCD systems could not be determined owing to the very low solubility of reserpine itself in water.

Conclusion

Three positional isomers of doubly branched βCDs, 1,*n*-$(G)_2$-βCDs were purified by HPLC on ODS column and a methanol–water system. The individual purification of 1,2-, 1,3-, and 1,4-isomers was not easy, while the mixture of the three isomers was relatively readily obtained. The low water-solubility of 1,4-isomer was a noteworthy characteristic among the three positional isomers. The doubly branched βCDs may be more suitable than non-branched βCD and mono-branched βCD, G-βCD, for safety *in vivo*, since their hemolytic activities are weaker than those of βCD and G-βCD. The complex-forming abilities of doubly branched βCDs for several poorly water-soluble guest compounds examined in this study were about the same as those of mono-branched βCD and non-branched βCD, and the extreme differences of those were not recognized among the three positional isomers of 1,*n*-$(G)_2$-βCDs. In contrast, the solubilities of guest compounds except reserpine were much more enhanced by doubly and mono-branched βCDs than by non-branched βCD.

In terms of hemolytic activity, solubility in water and in 10—50% (v/v) methanol aqueous solution, and solubilization of poorly water-soluble compounds, the use of 1,3-isomer is primarily recommended, and the mixture of the three positional isomers may also be useful.

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