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Enantioselective stabilization of inclusion complexes of metoprolol in carboxymethylated β-cyclodextrin

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday.

Abstract

The inclusion complexes of metoprolol (MT) and carboxymethyl-β-cyclodextrin (CMCD) were prepared and the stability constants of the complexes were determined. Binding studies performed using high performance liquid chromatography (HPLC), UV spectrometry and capillary electrophoresis (CE) indicated that a complex with 1:1 stoichiometry is predominant in the solution. The enantiomers of MT possess relatively high affinity towards CMCD with stability constants of 288 and 262 per M for (*R*)- and (*S*)-MT, respectively. Through nuclear magnetic resonance (NMR) analysis, MT was predicted to be a bent structure with phenyl ring of MT inserted in the shielding cavity of CMCD during complex formation. The NMR data suggested that the chiral side chain and the methoxyethyl moiety of MT are aligned in the deshielding zone, above and below the CMCD torus ring.

Keywords: Metoprolol; Enantioselectivity; β-Cyclodextrin; Stability constant; Nuclear magnetic resonance (NMR)

1. Introduction

Cyclodextrins (CDs) are oligoglycosides of six (α-CD), seven (β-CD) or eight (γ-CD) glucose units forming a ring with a cavity of moderate size. CDs as host molecules, can thus form inclusion complexes with various drug molecules as guest molecules and are utilized for improvement of drug properties [1] such as solubility, stability and bioavailability, etc. They are also optically active and offer the potential discrimination of enantiomeric substances. These characteristics of CDs permit them to be employed as stationary phase in gas and liquid chromatography [2,3] and as chiral additives in liquid chromatography [4] and capillary electrophoresis (CE) [5] for separation of drug enantiomers.

Most often, the discrimination of drug enantiomers involves the formation of hydrogen bonds

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between the CDs and molecules. The proximity of hydrogen bonding moieties can vary markedly for a pair of enantiomers with a concomitant difference in stability. Therefore, stability is a primary property of the complex that is created during the chiral discrimination process. The stability constant may also be defined using terms such as binding, complex or association constant. The comparison of differences in the stability constants, which characterize the interactions of host molecules with the analytes, allows a priori selection of the chiral selector. Thus, the determination of the stability constants is important in studying chiral separations and in their practical utilization [6].

Metoprolol (MT), 1-*iso*-propylamino-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol, is a β_1 selective adrenoceptor antagonist used for the treatment of angina and hypertension and is administered as a racemic mixture. The affinity of the β_1 adrenergic receptor for (*S*)-MT is significantly higher than for (*R*)-MT [7] and the enantiomers are metabolized at different rates [8]. Thus, stereoselective determination of MT is very important. Several reports have described the enantioselective synthesis [9,10] of MT and the separation and determination of its enantiomers using CE with modified β-CD as a chiral selector [11–13].

A number of different physicochemical methods have been described for the determination of the stability constants based on techniques such as solubility [14], spectrophotometric [15] and chromatographic methods [16,17]. NMR spectrometry was also used for host–guest interaction studies [18,19]. In MT there are three groups of diastereotopic protons in the vicinity of the asymmetric carbon atom. These protons should show in principle anisochronous signals in any achiral medium. In chiral medium, i.e. in chiral solvent or especially in a complexed form with chiral β-CD, the diastereotopic protons of each enantiomer may present more differentiated signals in NMR spectrum. This offers a considerable source of information for the study of enantioselective binding parameters such as the stoichiometry of the host–guest complexes and the apparent stability constant.

In this work, determination of the stability constant and prediction of the orientation of the MT molecule in carboxymethyl-β-cyclodextrin (CMCD) was carried out using UV spectrometry, HPLC, CE and NMR to evaluate the chiral recognition of MT enantiomers in CMCD.

2. Experimental

2.1. Apparatus

The experiments on CE were performed on a Biofocus 3000 CE system (Biorad, CA, USA) equipped with an autosampler, variable wavelength UV detector. Detection was performed at 210 nm. All separations were carried out on a 75 μm I.D. uncoated fused silica capillary (length 47, 40 cm to detector) with 10 kV of running voltage. The capillary was thermostated at 20 °C. Injection was performed hydrodynamically at the anodic end of the capillary with 3 psi × s. HPLC was consisted of LC-10AD pump, SPD-M10AVP diode array detector and CBM-10A system controller (Shimadzu Co., Japan). A reversed phase column, Spherisorb S3 ODS2 (2.0 × 50 mm, Waters, MA, USA), was used and thermostated at 30 °C with a column temperature controller (Waters). The flow rate of eluent was set 0.15 ml/min and the column effluent was monitored at 280 nm. ^{1}H-NMR spectra were obtained with Unity Inova-400 (Varian, CA, USA) spectrometer using D_2O as solvent. The spectra were obtained under the following conditions, acquisition time, 2.0 s; relaxation delay, 3.0 s; number of scan, 32; data points, 32 768; digital resolution, 0.244 Hz per point. Chemical shifts were referred to D_2O (4.81 ppm). An UV spectrophotometer (DU-650, Beckman, CA, USA) and a pH meter (ATI 370, Orion, MA, USA) were also used.

2.2. Chemicals and reagents

CMCD with average substitution degree of 0.5 were purchased from Wacker Chemie (Germany). Racemic MT and enantiomers were synthesized in this laboratory by the published method [9]. The optical purity of synthesized enantiomers, confi-

rmed by HPLC with Chiralcel OD (4.6 × 250 mm, Daicel Chem., Japan) as stationary phase, was more than 98%. Other chemicals and solvents were of analytical-reagent or HPLC grade.

2.3. Determination of stoichiometry

Before proceeding with the calculation of the stability constant, it is important that the stoichiometry of the complexes is calculated. A reliable determination of the complex stoichiometry is provided by the continuous variation technique (Job's plot). MT and CMCD were dissolved in deionized water at various molar ratios (1:9–9:1) and stirred to equilibrium for 12 h at room temperature. UV absorbance changes between free and complexed MT were measured at 284 nm and the stoichiometry was determined.

2.4. Determination of stability constants

HPLC, UV spectrometry and CE were used to determine the stability constants between MT and CMCD in aqueous solution. Determination of the stability constant was performed with reversed-phase HPLC using CMCD as a mobile phase additive. The mobile phase was composed of various concentrations of CMCD (0, 1, 3, 5, 8, 10 mM) in the mixed solution of acetate buffer (pH 5.0; 0.1 M) and methanol (80:20, v/v). The stability constant, K_c, was calculated by the conventional Scott's equation [20]:

$$\frac{1}{k'} = \frac{K_c}{K_D}[\mathrm{CD}] + \frac{1}{K_D} \quad (1)$$

where k' is the capacity factor of the peak of each enantiomer, [CD] is the concentration of CMCD in the mobile phase and K_D is a phase constant.

The differences in UV absorbance between free MT and complexed MT were used to determine the stability constant by UV spectrometry. MT (0.5 mM) was dissolved in acetate buffer (pH 4.0; 0.1 M) with increasing concentrations of CMCD (1, 4, 6, 10, 20, 30 mM) and stirred to equilibrium for 12 h at room temperature. UV absorbance changes between free and complexed MT were measured at 284 nm and the stability constants were calculated by the conventional Scott's equation as follows:

$$\frac{[\mathrm{MT}][\mathrm{CD}]}{d} = \frac{1}{K_c\varepsilon} + \frac{[\mathrm{CD}]}{\varepsilon} \quad (2)$$

where d is the change in UV absorbance between free and complexed MT and ε is the difference of molar absorptivity.

The stability constant was also determined by CE using an uncoated fused silica capillary. Before use, the capillary was washed successively with 0.1 M NaOH for 30 s, water for 2 min and separation buffer for 2 min. The running buffer was prepared by adding appropriate amounts of CMCD to acetate buffer (pH 4.0; 0.1 M) containing 5% 2-propanol as an organic modifier. The stability constant was calculated by first determining the mobility of the enantiomeric analyte and the complex using Eq. (3) [6]. This equation allowed the point-by-point calculation of the stability constant by a different method to that used in HPLC and UV spectrometry. Eq. (3) can be transformed into Eq. (4) by double reciprocal method [21]. This equation offers a linear plotting form, where the mobility of complexed MT is not required for calculation of stability constants.

$$K_c = \frac{1}{[\mathrm{CD}]}\frac{u_M - u_{eff,M}}{u_{eff,M} - u_{MC}} \quad (3)$$

$$\frac{1}{u_M - u_{eff,M}} = \frac{1}{u_M - u_{MC}K_c}\frac{1}{[\mathrm{CD}]} + \frac{1}{u_M - u_{MC}} \quad (4)$$

where u_M and u_{MC} are the ionic mobilities of free MT and complexed MT with CMCD, respectively. $u_{eff,M}$ is the effective mobility of MT and may be calculated from the migration time of analyte (t) and electroosmosis (t_o) at the used voltage (V), total (L) and detection length (l) of the capillary as shown in Eq. (5).

$$u_{eff} = \left(\frac{1}{t} - \frac{1}{t_o}\right)\frac{Ll}{V} \quad (5)$$

2.5. NMR measurement of inclusion complexes

The inclusion complexes for NMR study were prepared in deionized water using appropriate amounts of MT enantiomer and CMCD in a molar ratio of 1:1. The solution was stirred at room temperature for 24 h and lyophilized. The lyophilized complexes were then dissolved in D_2O and the NMR spectra were acquired. For the

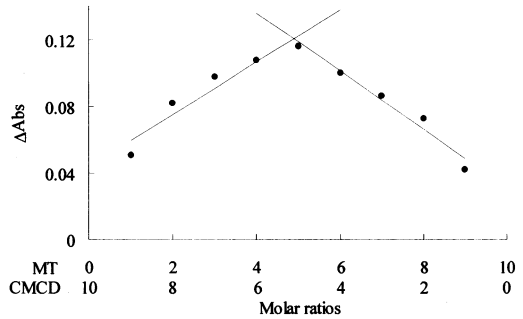

Fig. 1. Continuous variation plot for metoprolol and CMCD.

purpose of comparison pure MT in D_2O was also measured and the NMR signals and parameters were assigned from the spectrum of the protonated MT sample where 4 μl of concentrated HCl were added to 1 ml of solvent D_2O.

3. Results and discussion

3.1. Stoichiometry between MT and CMCD

The continuous variation plot of UV absorbance changes between free and complexed racemic MT (Fig. 1) shows that the changes in absorbance intensity are maximal when the molar fraction of racemic MT and CMCD is 5:5. This indicates that a complex with a 1:1 stoichiometry is predominant in the solution.

3.2. Stability constants between MT and CMCD

The retention times of MT enantiomers in HPLC decreased as the CMCD concentration, [CD], in the mobile phase was increased. The Scott's plot showed that there was good linear correlation between $1/k'$ and [CD] in HPLC (Fig. 2a). The equations of the lines for (*R*)- and (*S*)-MT were $y(1/k') = 12.9x$ ([CD]; M) + 0.036 ($r = 0.9995$) and $y = 11.8x + 0.036$ ($r = 0.9995$), respectively. The Scott's plot of [MT][CD]/*d* against [CD] by UV spectrometry also showed good linear correlation (Fig. 2b). The equations of the lines for (*R*)- and (*S*)-MT were y([MT][CD]/*d*; $M^2/A) = 1.26 \times 10^{-3}x$ ([CD]; M) + 5.18×10^{-6} ($r = 0.9994$) and $y = 1.36 \times 10^{-3}x + 5.89 \times 10^{-6}$ ($r = 0.9992$), respectively. The stability constants could be calculated from the equation as slope/intercept. The estimated stability constants (mean of K_R and K_S, stability constant of (*R*)- and (*S*)-MT, respectively) by HPLC and UV spectrometry were 347 per M ($K_R/K_S = 1.09$) and 238 per M ($K_R/K_S = 1.06$), respectively, which permitted the deduction of perceptible enantioselective inclusion of each enantiomer indicating the formation of a more stable complex of (*R*)-MT with CMCD. The difference in the values of the stability constants determined by HPLC and UV spectrometry may result from nonequivalence of solvents, concentration of CMCD, temperature, pH and other experimental parameters.

Using CE, the effective mobilities of MT ($u_{eff,M}$) in a electrolyte were calculated using Eq. (5) from

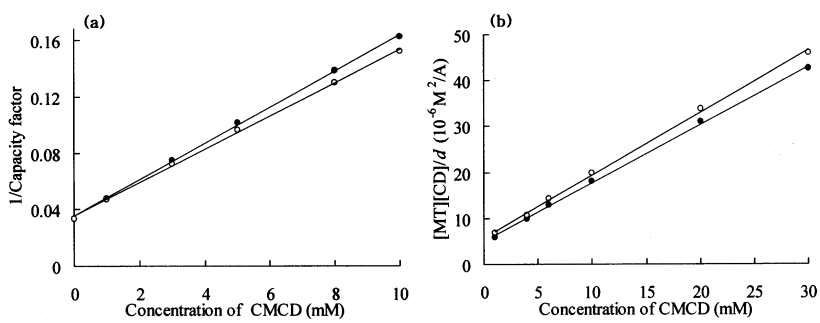

Fig. 2. Scott's plot for the determination of the stability constant between (*R*)-metoprolol (closed circle) or (*S*)-metoprolol (open circle) and CMCD by HPLC (a) and UV spectrometry (b). HPLC conditions, column; Spherisorb S3 ODS2 (2.0 × 150 mm), mobile phase; CMCD in mixed solution of acetate buffer (pH 5.0; 0.1 M) and methanol (80:20, v/v), flow rate; 0.15 ml/min.

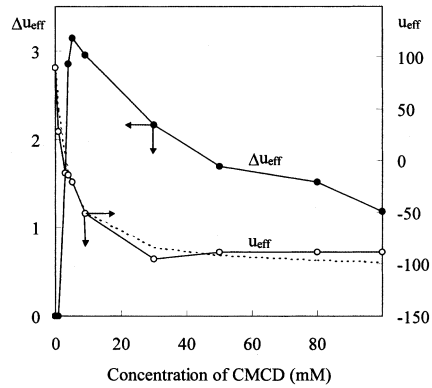

Fig. 3. The mobility of (*R*)-metoprolol (open circle) and the difference in mobility between the metoprolol enantiomers (closed circle) determined by CE as a function of CMCD concentration. Dashed line represents the approximate mobility of (*R*)-metoprolol by Lineweaver–Burk double reciprocal plot. Mobilities are given in 10^{-4} cm^2/V per min units. CE conditions, capillary; uncoated fused silica capillary, 75 μm I.D. × 47 cm (40 cm to detector), voltage; 10 kV, running buffer; CMCD in acetate buffer (pH 4.0; 0.1 M) containing 5% 2-propanol.

the migration time of MT and electroosmosis in a given concentration of CMCD. The u_M and u_{MC} are necessary to calculate the stability constants by Eq. (3). While u_M, the mobility of MT in the absence of the chiral selector in electrolyte, is easily accessible experimentally, u_{MC} could not be determined directly from experiments. The reason is that, strictly speaking, u_{MC} is the mobility of MT in the electrolyte containing an infinitely high concentration of the chiral selector. If the optimum concentration of chiral selector (C_{max}), which corresponds to a maximum difference in effective mobilities between two enantiomers, is known, u_{MC} can be calculated by Eq. (6).

$$u_{MC} = u_{eff,R,max} + u_{eff,S,max} - u_M \quad (6)$$

where $u_{eff,R,max}$ and $u_{eff,S,max}$ are the effective mobilities of (*R*)- and (*S*)-MT at optimum concentration of CMCD, respectively. Another way to estimate u_{MC} is the approximation of the limit approached by effective mobilities at increasing [CD].

As shown in Fig. 3, the maximum difference in mobility between (*R*)- and (*S*)-MT was at 5 mM CMCD ($C_{max} = 5$ mM). The mobilities of (*R*)- and (*S*)-MT at this concentration were -20 and -17 in 10^{-4} cm^2/V per min unit, respectively, which allowed the calculation of u_{MC} by Eq. (6) as -127 in 10^{-4} cm^2/V per min. The u_{MC} was also estimated using a Lineweaver-Burk double reciprocal plot indicated as a dashed line in Fig. 3. The approximated value was -105 in 10^{-4} cm^2/V per min. The u_{MC} value calculated by Eq. (6) was highly dependent on C_{max} value, which can be difficult to determine exactly. This difficulty has to be taken into account when estimating stability constants by CE. To avoid using an erroneous u_{MC} value, stability constants were calculated by Eq. (4) plotting $1/(u_M - u_{eff,M})$ versus 1/[CD]. The linear equations for (*R*)- and (*S*)-MT were $y(1/(u_M\text{-}u_{eff,M})$; /cm^2 V min) $= -0.178x$ (1/[CD]; per M) $- 5.28 \times 10^{-3}$ ($r = -0.9966$) and $y = -0.188x - 5.32 \times 10^{-3}$ ($r = -0.9965$), respectively. The stability constants could be calculated from the equation as intercept/slope.

The stability constants between MT and CMCD and enantioselectivity (K_R/K_S) values estimated by various methods are summarized in Table 1. Three different evaluation procedure calculating stability constants were applied to the same data obtained by CE. The values calculated by approximated u_{MC} were comparable with those by Eq. (4), which does not require the knowledge of u_{MC}. The largest value with the best enantioselectivity was obtained by HPLC. It is not always true that higher stability means better discrimination of enantiomers, but in this case higher enantioselectivity was achieved by larger stability constants. The differences in stability constants determined by various methods may result from different principles of measurement or nonequivalence of experimental conditions. In addition, CMCD used as chiral selector represents a multicomponent mixture, therefore, stability constants here can be considered not as absolutely correct values, but as apparent characteristics. Nevertheless, these averaged stability constants are very useful for understanding the mechanism of chiral recognition of CMCD.

Table 1
Stability constants (per M) of (*R*)- and (*S*)-metoprolol complexed in CMCD and enantioselectivity obtained by HPLC, UV spectrometry and CE

Complexed with	HPLC	UV	CE		
			Eq. (6)[a]	Approximately[b]	Eq. (4)[c]
(*R*)-metoprolol	362	244	234 ± 19	303 ± 20	298
(*S*)-metoprolol	332	231	223 ± 20	287 ± 18	283
α[d]	1.09	1.06	1.05	1.06	1.05

[a] Stability constants calculated with u_{MC}, derived by Eq. (6). Mean ± S.D. at four different concentrations (3, 4, 5 and 9 mM) of CMCD.
[b] Stability constants calculated with u_{MC}, by approximation using the Lineweaver–Burk equation. Mean ± S.D. at four different concentrations (3, 4, 5 and 9 mM) of CMCD.
[c] Stability constants calculated by double reciprocal linear plotting method.
[d] $\alpha = K_R/K_S$ for K_R and K_S, stability constants of (*R*)- and (*S*)-metoprolol, respectively.

3.3. NMR analysis of the MT–CMCD complex

MT, as shown in Fig. 4, has conformational flexibility, especially around the chiral side chain. The normal NMR spectrum of pure MT in D_2O, however, could not provide information about the absolute conformation, because of the poor solubility of MT in D_2O, and signal overlapping (Fig. 5a). Protonation of the amine group of MT improved the solubility, sensitivity and resolution of NMR spectrum. The ammonium ion also shifted the signals of neighboring protons to individually resolved positions (Fig. 5b). The assignment of chemical shifts and coupling constants of all protons were performed on this spectrum using an ACD/HNMR Spectrum Generator [22].

The chemical shifts show unequivocal diastereotopic separation of proton pairs 1a–1b, 3a–3b and 5a–5b in the neighborhood of the chiral center. With respect to the coupling pattern, it is interesting to note that the coupling constants of H^{1a} and H^{1b} to H^2 are identical ($J_{1a-2} = J_{1b-2} = 4.8$ Hz), while those of H^{3a} and H^{3b} to H^2 differ dramatically ($J_{3a-2} = 9.2$ Hz and $J_{3b-2} = 3.4$ Hz). Although the bonds C^1–C^2 and C^2–C^3 are known to be rotationally flexible, the only way to interpret this difference is with relation to vicinal coupling constant to the torsion dihedral angle. Referring to the Karplus relationship, the dihedral angles H^{1a}–C^1–C^2–H^2 and H^{1b}–C^1–C^2–H^2 must be around 60°, whereas the dihedral angle H^{3a}–C^3–C^2–H^2 is about 20° and H^{3b}–C^3–C^2–H^2 is about 90°. This 'fixed' conformation of MT is possible when the hydroxy and amine groups participate in an intermolecular hydrogen bond as H donor and/or acceptor. The chiral part of the MT can thus be predicted as bent structure with respect to the phenyl ring system.

From the NMR spectra of (*R*)-MT (Fig. 5c) and (*S*)-MT (Fig. 5d), complexed in CMCD, the chemical shifts were determined and compared with that of pure MT. This provided clear evidence of MT–CMCD interactions and the different behavior of (*R*)-MT and (*S*)-MT in complex formation. As the axial protons are normally more shielded than the equatorial protons in the cyclohexane system, the inner cavity and outside of the torus wall must be classified as the shielding zone and above and below the torus ring as the

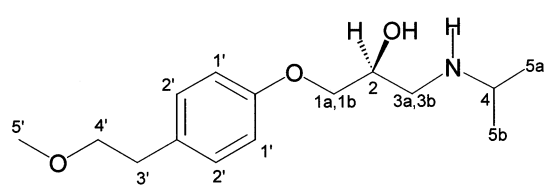

Fig. 4. Metoprolol with proton atoms numbered.

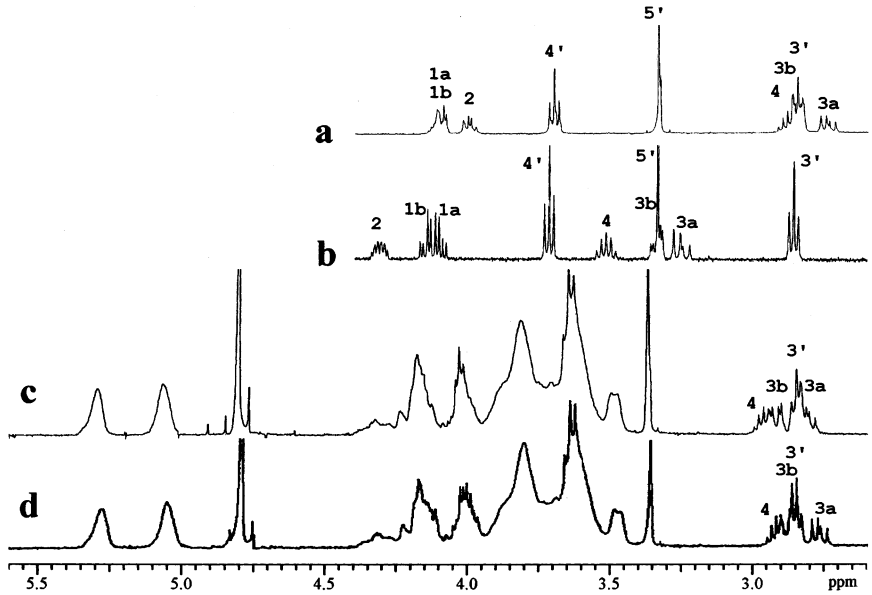

Fig. 5. NMR spectra of metoprolol (a), protonated metoprolol (b), (*R*)- (c) and (*S*)-metoprolol (d) complexed with CMCD.

deshielding zone. The chemical shifts of $H^{1'}$and $H^{2'}$ in the phenyl ring of MT–CMCD complex are shifted to a higher field compared with pure MT in both enantiomers ($\Delta\delta = 0.06$ ppm). It is known that the phenyl ring must be inserted in the hydrophobic inner cavity in complex formation with CD and the NMR data concurs with this observation. Furthermore the direct neighboring $H^{3'}$'s, which are probably on the boundary between the shielding and deshielding zone, stay at the same position ($\Delta\delta < 0.01$ ppm) and the $H^{4'}$'s, which get into the shielding zone due to the bent structure, shifted ($\Delta\delta = 0.05$ ppm) the same amount as $H^{1'}$and $H^{2'}$. The methoxy group must then be clearly in the deshielding zone below the torus ring. With these findings the total structure of MT–CMCD complex can be predicted (Fig. 6).

The increase in chemical shifts of protons around the chiral center is twice as large in (*R*)-MT–CMCD ($\Delta\delta = 0.062$ ppm) as in (*S*)-MT–CMCD ($\Delta\delta = 0.034$ ppm), while the changes of other protons are almost the same in both enantiomers. This is because the chiral group of (*R*)-MT can bind more closely to the deshielding region above the torus ring than the (*S*)-MT.

4. Conclusions

The chiral recognition of MT enantiomers in CMCD was evaluated using the stability constants and the structure of inclusion complex between MT and CMCD. The stability constants determined by UV spectrometry, HPLC and CE

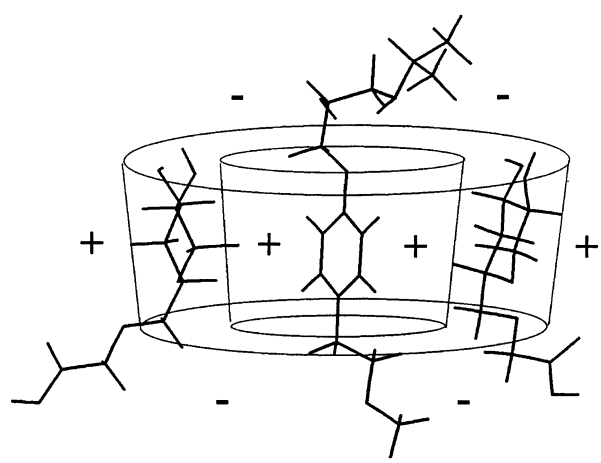

Fig. 6. A proposed structure of (*R*)-metoprolol–CMCD complex. Only the first and fourth glucose units of CMCD were drawn explicitly and (+) and (−) denote the magnetic shielding and deshielding zone, respectively.

permitted the deduction of perceptible enantioselective inclusion of each enantiomer, and indicated the formation of a more stable complex of (*R*)-MT than (*S*)-MT with CMCD. Though there were appreciable differences in the magnitude of the stability constants from different methods, the determined values provided useful information on the chiral recognition of MT in CMCD.

NMR analysis was consistent with a bent structure for MT with the chiral side chain and the methoxyethyl moiety of MT aligned in the deshielding zone, above and below the CMCD torus ring, respectively. The increase in the chemical shifts of the protons around the chiral center of (*R*)-MT in CMCD is larger than that of (*S*)-MT, consequently the chiral group of (*R*)-MT can bind more closely to the deshielding region of the torus ring than (*S*)-MT. The data from the NMR study agreed with the stability constants derived by UV spectrometry, HPLC and CE.

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