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Mechanistic study of enantiomeric recognition with native γ-cyclodextrin by capillary electrophoresis, reversed-phase liquid chromatography, nuclear magnetic resonance spectroscopy, electrospray mass spectrometry and circular dichroism techniques

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Abstract

The possible mechanisms for the chiral recognition of 2(*S*)-(3,5-bis-trifluoromethyl-phenyl)-2-[3(*S*)-(4-fluorophenyl)-4-(1H-[1,2,4]triazol-3-ylmethyl)-morpholin-2(*R*)-yloxy]-ethanol (compound A) and its enantiomer with native γ-cyclodextrin (γ-CD) were investigated using capillary electrophoresis (CE), reversed-phase liquid chromatography (RPLC), proton (^{1}H), fluorine (^{19}F) and carbon (^{13}C) nuclear magnetic resonance spectroscopy (NMR), electrospray mass spectrometry (ESI-MS) and circular dichroism (CD). All experiments provided clear evidence of the formation of diastereomeric complexes between the enantiomers and γ-CD. Proton, fluorine and carbon NMR spectra suggested that both aromatic rings, with mono-fluoro and bis-tri-fluoro functional groups, on the guest molecule were partially included into the cavity of the γ-CD. ESI-MS spectra indicated that the diastereomeric complexes have a 1:1 stoichiometric ratio. The binding constants of the diastereomeric complexes obtained by CE, RPLC and CD were compared. The effects of the γ-CD concentration, organic modifiers and temperature on the CE-chiral separation were also investigated.
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Keywords: Chiral recognition; Circular dichroism; Nuclear magnetic resonance spectroscopy; Electrospray mass spectrometry; γ-Cyclodextrin

1. Introduction

The analysis of enantiomeric compounds is a very important field, especially in the pharmaceutical industry, as potentially the two enantiomers may possess different pharmacological properties. In the past two decades, the increased attention to development of chirally pure compounds in the pharmaceutical industry has stimulated method development and mechanistic investigations of enantiomeric separations. Enantiomeric separation can be achieved through various techniques such as liquid chromatography (LC) and capillary electrophoresis (CE). The separation mechanisms and applications of both chiral LC and CE have been extensively covered in recent publications [1–7]. In addition, various spectroscopic techniques, such as electron-spin resonance (ESR) [8,9], nuclear magnetic resonance (NMR) [10–14], ultraviolet (UV) [15,16], circular dichroism (CD) [15,17,18], optical rotatory dispersion (ORD) [19], electrospray ionization mass spec-

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trometry (ESI-MS) [20,21] have also been used in order to understand interactions between the chiral selectors and the selectants. Among these techniques, NMR is considered to be the most powerful tool for structural elucidation at the molecular level. In the last few years, ESI-MS has become a popular tool for investigation of solution complexation since it is the mildest ionization method for mass spectrometry, thus the 'softness' of ESI allows the detection of non-covalently bonded complexes. CD is a simple and useful tool in determining the formation constant of complexation if an induced CD spectrum is available.

Chiral separations can only take place within a chiral environment. For this purpose, the presence of a chiral selector is necessary. Cyclodextrins are the most widely used chiral selectors in CE, and the cyclodextrin-bonded stationary phases were the first CSP deliberately designed to be used in the RPLC mode [22]. Several mathematic models have been established, particularly in CE, to describe the behavior of cyclodextrins when used as chiral selectors. Wren and Rowe proposed the first model [23,24], which allows for the optimization as a function of concentration of the cyclodextrin through maximization of the mobility difference between the enantiomers. Goodall and co-workers refined this model and showed how to determine binding constants from CE mobility data [25]. Vigh and co-workers extended the model to explicitly include the effects of pH and the concentration of a chiral selector (duoselectivity chiral separation model) [26]. On the other hand, Chankvetadze and co-workers have published a series of papers to describe structural information of selector-selectant complexes in CE solution using NMR, ESI-MS and other spectroscopic techniques [3,11–13]. They demonstrated the predictive character of NMR data for the optimization of chiral CE parameters.

In this paper, mechanistic aspects of enantiomeric recognition with native γ-CD for 2(*S*)-(3,5-bis-trifluoromethyl-phenyl)-2-[3(*S*)-(4-fluorophenyl)-4-(1H-[1,2,4]triazol-3-ylmethyl)-morpholin-2(*R*)-yl-oxy]-ethanol (compound A as shown in Fig. 1) and its enantiomer (compound A′) are investigated using CE, RPLC, NMR, ESI-MS and CD techniques. Binding constants of the diastereomeric complexes obtained by CE, RPLC and CD were compared. The effects of the γ-CD concentration, organic modifiers and temperature on the CE-chiral separation are also presented.

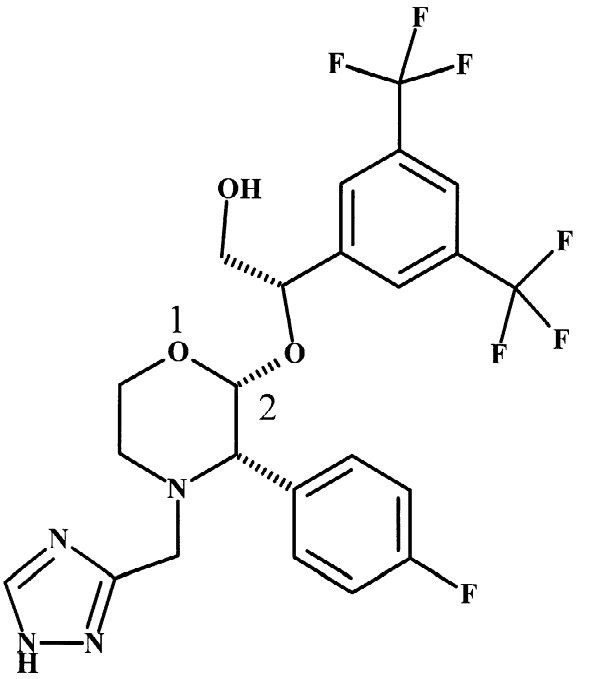

Fig. 1. Chemical structures of the compound A. The molecular mass is 534.4.

2. Experimental

2.1. Instrumentation

In the CE mode, a Hewlett-Packard ^{3}D CE (Hewlett-Packard, Wilmington, DE, USA) system was used. In the RPLC mode, a Hewlett-Packard LC 1100 (Hewlett-Packard) system was used. Data collection and analysis were performed with a PE Nelson data system equipped with Turbochrom Chrom software (PE Nelson, Cupertino, CA, USA). For CD studies, a Jasco-810 spectropolarimeter (Japan Spectroscopic CD, Japan) with a 1-cm thick quartz cell was used. For ESI-MS, an ion trap mass spectrometer (LCQ, Finnigan, Branford, CT, USA) equipped with an electrospray interface was used. The detection was performed in the positive ion mode. The ionization voltage was 6 kV for all

samples. The temperature of the inlet capillary was 200 °C. Each sample was introduced into the ion source with a flow-rate of 5 μl/min using a syringe pump. For the NMR studies, an AVANCE DRX-400 system (Brüker, MA, USA) was used. The frequency was set at 399.9 MHZ for ^{1}H, 376.2 for ^{19}F, and 100 MHZ for ^{13}C.

2.2. Reagents

Phosphate buffers (50 m*M* with different pH), 1 and 0.1 N sodium hydroxide (NaOH) were purchased from Agilent Technologies (Wilmington, DE, USA). Methanol (MeOH) and acetonitrile (MeCN) were obtained from Fisher Scientific (Springfield, NJ, USA). Deuterium oxide (D_2O), acetonitrile-d_3, formic acid-d_2 and 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS) were purchased from Isotec (Miamisburg, OH, USA). γ-Cyclodextrin (γ-CD) and acetone were obtained from Aldrich (Milwaukee, WI, USA). Deionized (D.I.) water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Chromatographic capillary and columns

The columns used in RPLC mode were YMC-C_4 (Waters, Millipore, Bedford, MA, USA), Phenomenex-C_5 (Phenomenex, CA, USA), Zorbax Rx C_8, Zorbax CN (Agilent Technologies) and Cyclobond-ii (Advance Separation Technologies, Whippany, NJ, USA). All columns were 15 cm in length and 4.6 mm in I.D. with a particle size at 5 μm. The capillary used in CE was fused-silica with 75 μm I.D. and with two different lengths (64.5 and 33.5 cm, respectively) (Agilent Technologies)

2.4. Chromatographic conditions

All LC separations, except where specified, were performed at a temperature of 25 °C. The mobile phases were isocratically pre-pump-mixed at specified compositions. The flow-rate was 1.0 ml/min, the injection volume was 10 μl; the detection was UV at 215 nm. The capacity factor k' was determined as $k' = (t_R - t_0)/t_0$, where t_R and t_0 were the retention times of retained and unretained compounds, respectively. In RPLC, t_0 was determined by the solvent peak. In CZE, except when systematically varying the composition, the background electrolyte (BGE) used was a 25 m*M* sodium phosphate buffer (pH 2.5). In all cases, a diode array detector was operated at 200 nm. Hydrodynamic sample injection was used with a 3-s injection time at a 50-mbar pressure. The applied voltage and capillary temperature were varied. The EOF was determined by acetone as a neutral marker. A nonlinear least-squares program written in C++ combined with a SAS statistical software package was used to calculate the binding constants based on the CE data.

3. Results and discussion

3.1. CE studies

3.1.1. Effect of γ-CD concentration

The concentration of γ-CD ([γ-CD]) was varied from 0 to 50 m*M*. As shown in Fig. 2a,b, there is no difference in the effective mobilities (μ) of the two enantiomers with no chiral selector present. As [γ-CD] was increased, μ (viscosity-corrected) decreased due to the complexation.

In previous studies, we have pointed out that for a basic enantiomer at low pH BGE, the mathematical equations based on Vigh's duoselective model and Wren–Rowe's model are essentially the same [27,28]. Therefore, the bonding constants for compound A and its enantiomer (compound A′) can be determined based on Wren–Rowe's equation as shown below:

$$KC = \frac{\mu_0 - \mu}{\mu - \mu_\infty}$$

where μ_0 is the mobility of the uncomplexed compound (measured at $C = 0$), μ_∞ is that of the completely complexed enantiomer, and K is the binding constant for the complex.

In Fig. 2a, the points are the experimental effective mobilities that have been corrected for the change of the viscosity. From a non-linear fit, the binding constants were determined as $K_A = 120.0 \pm 9.1\ M^{-1}$ and $K_{A'} = 37.5 \pm 3.8\ M^{-1}$. The uncertainties (standard error in the K values) are about 10–15%. The larger binding constant of compound

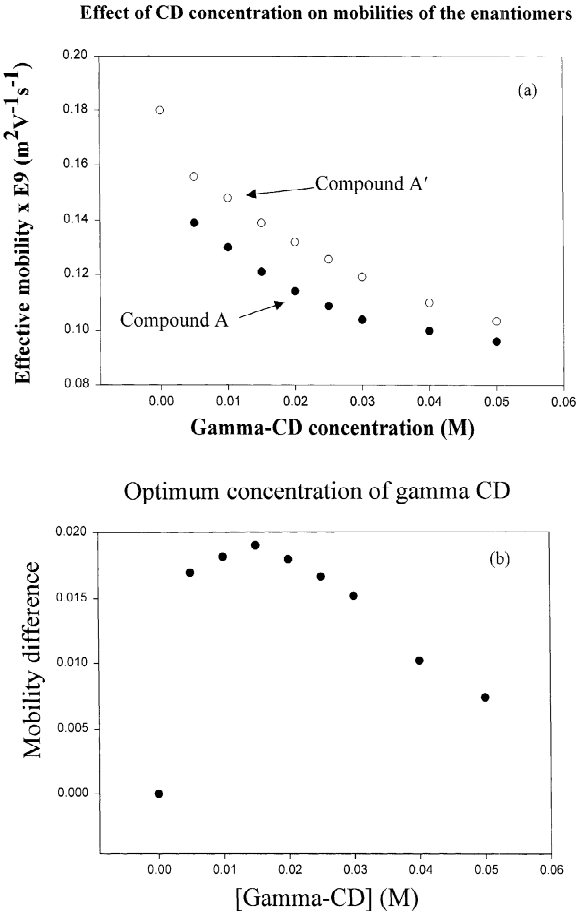

Fig. 2. Effect of [γ-CD] on effective mobilities of compound A and its enantiomer (a); effect of [γ-CD] on mobility difference (b). Conditions: the running buffer was 25 m*M* sodium phosphate buffer (pH 2.5), a positive 15-kV voltage was applied, the capillary was fused-silica, 64.5 cm in length and 75 μm in internal diameter. The capillary temperature was maintained at 25 °C, and the UV wavelength was set at 200 nm. The concentration of γ-CD was varied. The sample solution was a 0.5 mg/ml racemic of compound A.

A indicates it complexes with γ-CD more strongly than its enantiomer does.

Wren–Rowe predicted that the μ_∞ values of the two enantiomers would be identical, and it was proved in the experiment of Penn et al. [25]. Therefore the difference in enantiomer mobilities, $\Delta\mu$, can be derived from the above equation and an optimum C_{op} in $\Delta\mu_{max}$ can be expressed as $C_{op} = (K_A K_{A'})^{-1/2}$, where K_A and $K_{A'}$ represent binding constants for the enantiomeric compounds A and A′, respectively. Based on the derived binding constant of the compound A and A′, the C_{op} of γ-CD is calculated to be 15 m*M*. The $\Delta\mu$–*C* curve (Fig. 2b) showed a good agreement with the predicted value.

3.1.2. Effect of the organic modifiers

The addition of organic modifiers to the BGE can alter the selectivity. Several groups have reported that the addition of organic modifiers such as methanol and acetonitrile decreases the magnitude of the CD-enantiomer binding constants [5,16,27,29,30], The common explanation for this is that the organic modifier competes with the guest molecule and reduces the affinity of the analyte for the CD cavity. In addition, it is also recognized that organic modifiers can effect the EOF, the viscosity, dielectric constant and conductivity of the BGE, hence effecting the separation. In the present study, the effect on the enantioseparation of compound A and its enantiomer were investigated using two common organic solvents, acetonitrile (MeCN) and methanol (MeOH). As can be seen from Fig. 3, both migration time and resolution were changed as concentrations of acetonitrile were increased. Similar behavior was also observed when MeOH was added into the BGE. To substantiate the cause of the effect of the organic modifier, additional experiments were carried out using a BGE containing 30% MECN or MeOH. The

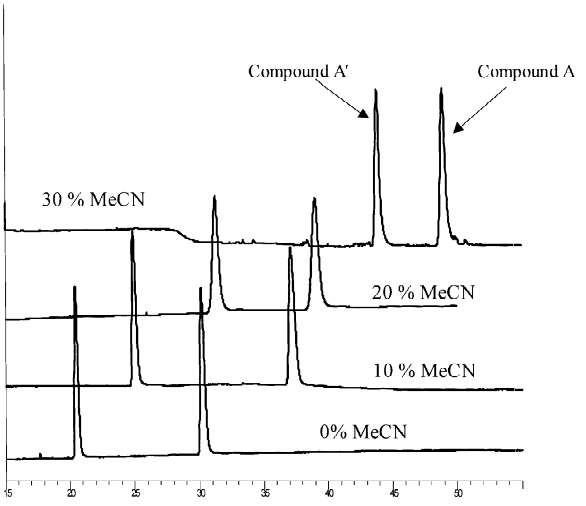

Fig. 3. Electropherograms of compound A and its enantiomer (compound A′) with various concentrations of the MeCN. Conditions are the same as in Fig. 2 except the concentrations of MeCN were varied and the capillary temperature was maintained at 20 °C.

Table 1
Effect of the organic modifiers on binding constant of [compound A: KCD] complex

Organic solvents	K_A (M^{-1})	$K_{A'}$ (M^{-1})
No organic solvent	120.0±15.2	37.4±5.2
MeCN	37.2±5.3	12.3±1.5
MeOH	45.6±6.3	15.6±1.5

The conditions are the same as in Fig. 2, except the concentration of MeCN(or MeOH) was fixed at 30%.

concentration of γ-CD in BGE was varied in order to determine the binding constants from the nonlinear least square fit of the data to Wren–Rowe's equation. The derived binding constants are listed in Table 1. As expected, the binding constants decrease with increasing organic concentrations. Acetonitrile has a greater influence than methanol.

3.1.3. Effect of other parameters

Many other parameters, such as temperature, capillary length and the pH of the BGE, can also effect the CE-chiral separation. The effect of temperature was investigated from 10 to 60 °C at 10 °C intervals. The relationship of current versus temperature was linear indicating that there was no excessive joule heating. As shown in Fig. 4, the enantioresolutions of compound A and its enantiomer were decreased as the temperature increased with a shorter run time. The plots of the ln α ($\alpha = \mu_A / \mu_{A'}$ where μ_A and $\mu_{A'}$ are the effective mobilities of compound A and A′, respectively) versus $1/T$ were linear with r^2 0.999 for both peaks. This behavior is consistent with that described in the literature [27,28,31].

Compound A has a pK_a of 3.8. Enantioseparation decreased as the pH increased and was completely lost at a pH of 6.0, as the analytes converted into their neutral form and co-eluted with the EOF. The effect of the capillary length and the applied electric field can also be observed from Figs. 3 and 4, when other conditions were kept the same. It was found

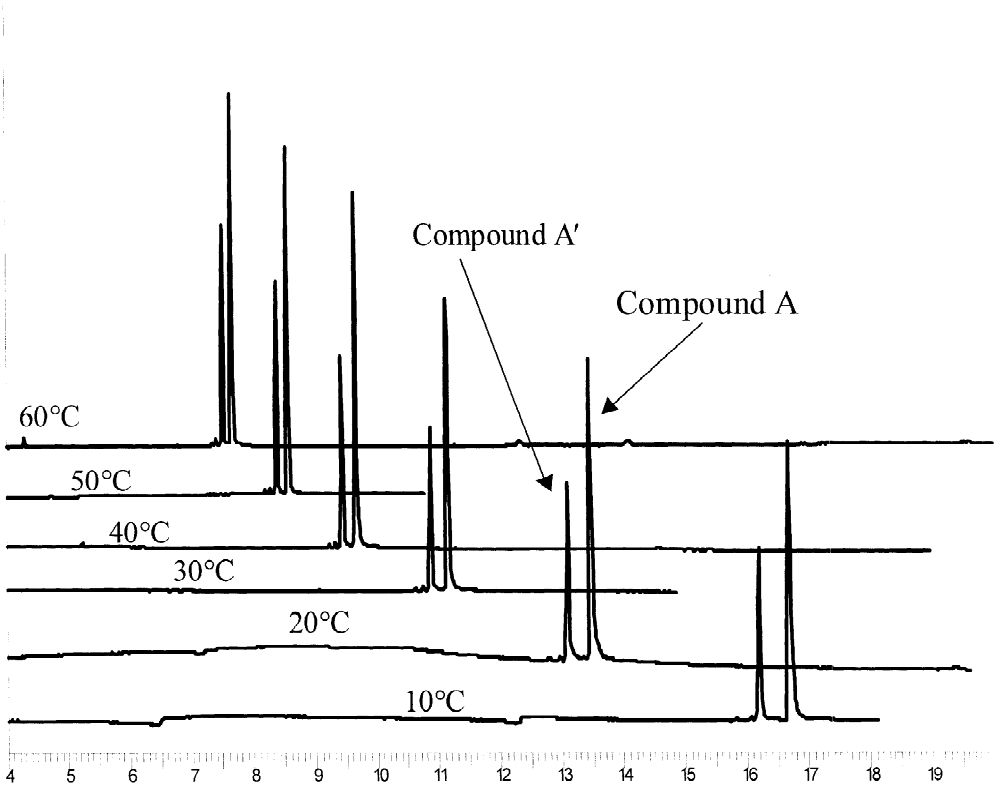

Fig. 4. Electropherograms of compound A and its enantiomer at different temperatures. Conditions: the running buffer was a 25 m*M* sodium phosphate buffer (pH 2.5), a positive 20-kV voltage was applied, the capillary was fused-silica, 33.5 cm in length and 75 μm in internal diameter. The capillary temperature was varied from 10 to 60 °C, and the UV wavelength was set at 200 nm. The concentration of γ-CD was 15 m*M*. The sample solution was a 0.5-mg/ml racemate of compound A spiked with compound A.

that the shorter the capillary length, the shorter the run time and the higher the applied electric field, the shorter the run-time, with sacrifices made in the resolution.

3.2. RPLC

Two different uses of cyclodextrins have emerged in RPLC. Cyclodextrins have been bonded to silica to form chiral stationary phases and have also been used as chiral mobile-phase additives. The chiral recognition of compound A and its enantiomer were examined by using both approaches. The first approach was carried out using a chiral column with a γ-CD stationary phase (Cyclobond II column) and different mobile phases. No enantioseparation was observed using a low pH (2.5) buffer. Enantiomeric separations were achieved with mobile phases containing an 80:20 ratio of A/B (A, 15 m*M* Na_2HPO_4; B, MeOH or MeCN), when the pH of mobile phase A was adjusted to 7.0. This is in contrast to the CE separation due to different separation mechanisms. In CE, the separation is based on the difference in the charge–size ratio of analytes. In RPLC, the separation is mainly based on the hydrophobic interaction between the analytes and the stationary phase. As mentioned in the previous sections, compound A is a very weak base with a pK_a of 3.8, turning neutral as the pH increases, thus the enantioseparation is lost because the γ-CD itself is also neutral and all peaks would be co-eluted with EOF. However, in RPLC with a γ-CD stationary phase, the neutral form of compound A and its enantiomer resulted in more hydrophobic interactions with the stationary phase, hence enhancing the separation. A comparison of chromatograms is displayed in Fig. 5.

The second approach was conducted by adding γ-CD into a mobile phase containing a 70:30 ratio of A/B (A, 25 m*M* sodium phosphate buffer, the pH of the buffers were adjusted to 2.5 and 7.0, respectively; B, MeCN). The concentrations of γ-CD were varied from 5 to 35 m*M*. Various RPLC columns, such as C_4, C_5, C_8, C_{18} were screened with different mobile phase ratios. No enantioseparation was observed under any of the screened conditions. However, it was noticed that the capacity (k') decreased with increasing concentration of γ-CD on each selected column at both low and high pH mobile phases. The results suggested compound A formed a complex with γ-CD, but the differences in the

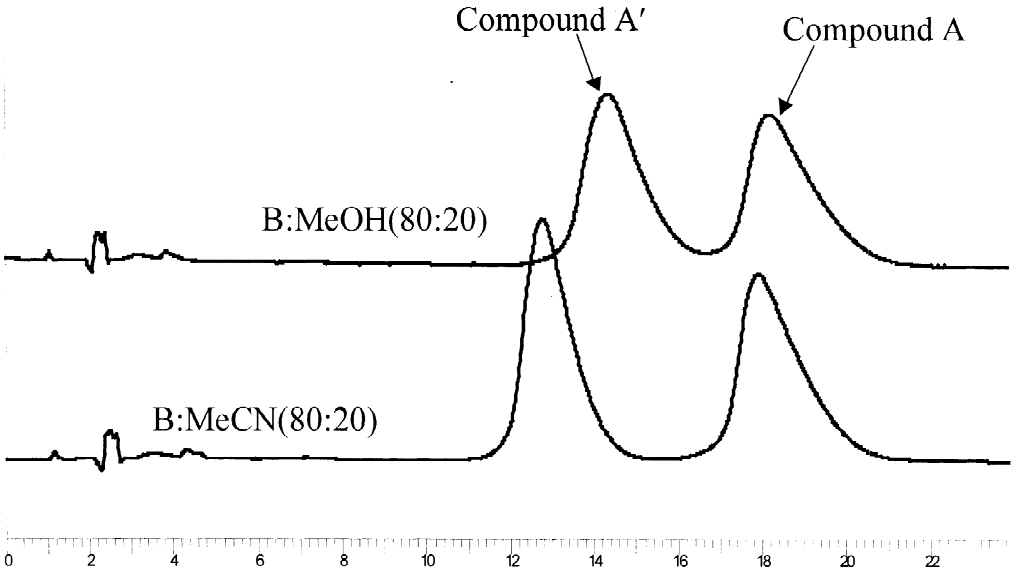

Fig. 5. Chiral separation of compound A and its enantiomer by a γ-CD stationary phase. Conditions: column was Cyclobond II, the mobile phase A was a 25 m*M* phosphate buffer (pH 7.0), the mobile phase B was ACN (or MeOH), the mobile phase ratio was 80:20 (v/v), the flow-rate was 1.0 ml/min, the column temperature was maintained at 25 °C, and the UV wavelength was set at 215 nm. The injection volume was 10 μl. The sample solution was a 0.5-mg/ml racemate of compound A.

adsorption of diastereomeric complexes on the surface of the hydrophobic stationary phase are not large enough to result in a chiral separation.

Mohseni and Hurtubise proposed the following equation based on a 1:1 guest-host equilibrium [32]:

$$1/k' = 1/k'_0 + K_f/k'_0[CS],$$

where k' is the capacity factor, [CS] is the chiral selector concentration in the mobile phase, k'_0 is the capacity factor of the analyte in the absence of the chiral selector, and K_f is the apparent formation constant which may be calculated based on a linear plot of $[1/k']$–$[CD]$. When the reciprocal of k' of a racemate of compound A obtained from a YMC-C_4 column was plotted versus γ-CD concentrations, a straight line with linearity $R^2 > 0.99$ was obtained. These results suggested a 1:1 guest–CD complex is preferred [33]. The binding constant calculated based on the plot is similar to that obtained from CE determinations under similar BGE conditions (15.5 vs. 37.3) considering the different experimental modes.

3.3. Circular dichroism spectroscopy

The changes of the ellipticities of compound A were measured as a function of γ-CD. The concentration of compound A was held constant at 7.5×10^{-5} *M*, and the circular dichroism spectra were collected at γ-CD concentrations of 0.0, 3.9×10^{-3}, 7.7×10^{-3}, 1.2×10^{-2} and 1.5 x10^{-2} *M*. As can be seen from Fig. 6, CD spectra of compound A in the absence of γ-CD gives a negative CD band at 217.3 nm. It appears that the peak results from the $\pi-\pi^*$ transition of triazole coupling with $\pi-\pi^*$ transitions of aromatic rings since these transitions are close in wavelength and space for a chiral exciton interaction [34]. The intensity of the CD peak becomes lower with increasing γ-CD concentrations and the λ_{max} of CD band was slightly shifted from 217.3 to 218.1 nm. For a 1:1 complexation, the binding constant can be determined based on a modified Benesi–Hildebrand equation as described below [16]:

$$(\Delta\theta_{obs})^{-1} = (K\,\Delta\theta_C)^{-1}[B]^{-1} + (\Delta\theta_C)^{-1}$$

where $\Delta\theta_{obs}$ is the observed ellipticity of the analyte

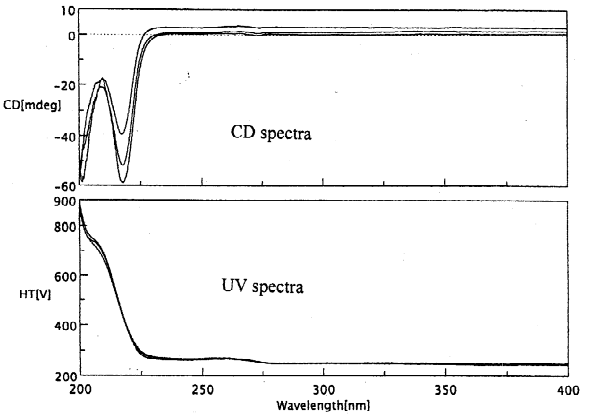

Fig. 6. Example of UV and CD spectra of compound A at various γ-CD concentration ranging from 0.0 to 1.5×10^{-2} *M*, read from the bottom to top. The concentration of the compound A was 7.5×10^{-5} *M*. The temperature was 25 °C.

at different γ-CD concentrations, and $\Delta\theta_C$ is the ellipticity difference before and after complexations. [B] is the molar concentration of the γ-CD and K is the binding constant. The data collected from the CD spectra were well-fitted into the $(\Delta\theta_{obs})^{-1}$–$[B]^{-1}$ plot with a linearity (R^2) of 0.999. A binding constant was determined to be 123.3±10 by dividing the intercept by the slope This value is in good agreement with the CE determination.

3.4. ESI-MS

Chiral recognition of compound A and its enantiomer was examined by applying ESI-MS to mixtures of γ-CD and compound A. Fig. 7a provides direct evidence of a 1:1 stoichiometric ratio of the host–guest complex. Other stoichiometries, such as 2:1, were not observed from the MS spectrum due to the limitation of the maximum mass range of the LCQ-MS instrument. However, the experimental data for the binding constant determinations by CE, LC and CD fit the 1:1 equilibrium models very well, and agree with ESI-MS data.

Similar ESI-MS spectra were obtained for a mixture of γ-CD and a racemic mixture of compound A, but the intensity of the complex signal was lower. Hercules examined the strength of chiral recognition based on the relative intensity ratio ($R = I$[complex]$/I$[host]) of complex to host, where I is

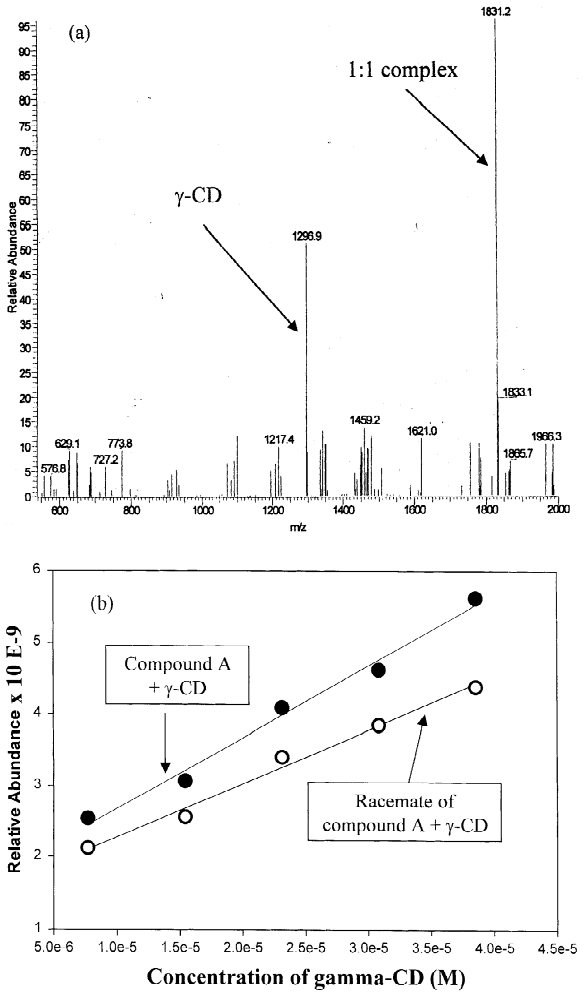

Fig. 7. A typical mass spectrum of [γ-CD:compound A] complex at a 1:1 ratio (a), the effect of γ-CD concentration on the intensities of the complexes signal (b). Conditions: the detection was performed in the positive ion mode. The ionization voltage was 6 kV for all samples. The temperature of the inlet capillary was 200 °C. Each sample was introduced into the ion source with a flow-rate of 5 μl/min using a syringe pump and the average intensity was collected over an 1 min range in duplication. The concentration of the guest compound (compound A or its racemic mixture) was 7.5×10^{-6} *M*. The γ-CD concentration was varied. The diluent for all sample solution was ACN–0.1% formic acid at 20:80 (v/v) ratio.

intensity. They concluded that the higher the relative intensity, the stronger the complex binding between host and guest [21]. To confirm their conclusion, a more accurate calibration experiment was performed in the present study. Two calibration curves of intensities of molecular ion signals ([compound A:γ-CD] and [racemate of compound A:γ-CD] complexes) at various γ-CD concentrations were constructed as shown in Fig. 7b. The experiments were carried out by changing the concentration of γ-CD from $7.7\times\ 10^{-6}$ to 3.9×10^{-5} *M* while keeping the concentration of compound A or its racemic solution at 7.5×10^{-6} *M*. As can be seen from Fig. 7b, the intensity of the complex signal in a mixture of compound A with γ-CD is consistently higher than in a mixture of the racemate of compound A with γ-CD. The linearity R^2 values of both plots are greater than 0.98. The average relative intensity ratio (*R*) was determined to be 2.53 ± 0.05 for compound A and 1.85 ± 0.06 for the racemate of compound A. These results indicated that γ-CD binds compound A more strongly than its enantiomer, which is consistent with CE results.

3.5. NMR

NMR is one of the most powerful tools for elucidation of structural information. The use of NMR to study the chiral recognition of enantiomeric compounds with cyclodextrins is well established [1,3,11–13]. The changes in chemical shifts of both guest and host upon complexation, defined as complexation-induced chemical shift (CICS), as well as signal splitting of the racemic guest, defined as complexation-induced chemical shift nonequivalence (CICSN), may provide information about chiral recognition at the molecular level.

3.5.1. ^1H NMR

Stock solutions of compound A, racemate of compound A and γ-CD were prepared in a mixture of acetonitrile-d_3/0.1% of formic acid-d_2 (80:20, v/v). Compound A or its racemate were mixed with γ-CD at 1:1 molar ratios, respectively. Small amounts of DSS were added into each working solution. The 400 MHz ^{1}H NMR spectra were collected for free compound A and its racemate, in the absence and presence of γ-CD. All ^{1}H chemical shifts were referenced to the DSS signal at 0 ppm. As expected, the chemical shift of free compound A and its racemate were the same since they possessed the same physical and chemical properties. However, CICS were observed for compound A and its

racemic mixture and CICSN was seen for the racemic mixture of compound A in the presence of γ-CD. The magnitudes of CICS are quite small. The shift difference ($\Delta\delta_{CICS}$) between free guest and complex spectra were calculated by subtracting the chemical shift of free compound A from the CICS of complexes. $\Delta\delta$ values that are equal to or greater than 0.01 ppm are listed in Table 2. These results suggested that several functional groups on compound A, including triazole, morpholine, mono-fluoro and bis-tris-fluoro phenyl rings, were involved in the complexation. The interaction could be a result of hydrophobic interaction (e.g., between phenyl ring and γ-CD cavity) or hydrogen bonding (e.g., between triazole ring and hydroxy group on the exterior of γ-CD). Further evidence can be viewed clearly from Fig. 8a. The resonance signal of H2-proton on the morpholine moiety at 4.68 ppm was split into two peaks upon the addition of γ-CD into the racemic mixture of compound A. This observation revealed an enantiospecific interaction through diastereomeric complexation. Since no ^{1}H NMR signal splitting was observed for both aromatic rings of the racemic mixture in the presence of γ-CD, further investigation was carried out using ^{19}F NMR.

3.5.2. ^{19}F NMR

The same solutions used for the ^{1}H NMR experiments were also evaluated by ^{19}F NMR. Two singlets were observed for the free compound A (or its racemic mixture) at approximately −63.2 and −112.1 ppm. They represent the bis-tri-fluoro and mono-fluoro moieties on the aromatic rings, respectively. Upon the addition of γ-CD into compound A, CICS was observed for both signals. This suggested that the complex was formed by inclusion of the mono-fluoro and bis-tris-fluoro phenyl rings into the cavity of γ-CD. The most pronounced evidence can be viewed clearly from the Fig. 8b. Upon the addition of γ-CD into the racemic mixture of compound A, both singlets of the mono-fluoro and the bis-tris-fluoro groups split into two peaks corresponding to each enantiomer. A baseline resolution of split peaks was observed for the mono-fluoro moiety. The peaks were identified, as shown in Fig. 8b, by spiking compound A into the racemic mixture. The downfield CICS for compound A is larger than for its enantiomer for both mono-fluoro and bis-tri-fluoro moieties. This indicated that compound A is more favorable to complex with γ-CD and this is consistent with CE, LC and ESI-MS determinations.

Table 2
Proton complexation-induced chemical shift (CICS) differences ($\Delta\delta_{CICS}$) of compound A with γ-CD

δ (ppm) of free compound A	δ_{CICS} (ppm) of mix of compound A and γ-CD at 1:1 ratio	$\Delta\delta_{CICS}$ ($=\delta_{CICS}-\delta$)
8.53	8.52	−0.01
7.62	7.60	−0.02
5.02	5.01	−0.01
5.01	5.00	−0.01
4.68	4.70	0.02
4.51	4.50	−0.01
4.48	4.47	−0.01
4.45	4.44	−0.01
4.40	4.39	−0.01
4.26	4.25	−0.01
4.03	4.02	−0.01

The conditions are the same as in Fig. 8.

3.5.3. ^{13}C NMR

Since the CICS assessment was not trivial for the γ-CD by ^{1}H NMR due to the overlapping of the selector and selectand signals, ^{13}C NMR was also utilized. The shift differences ($\Delta\delta_{CICS}$) of each carbon on γ-CD between free γ-CD and complex spectra are listed in Table 3. It is well known that the interior of γ-CD is relatively hydrophobic due to the presence of C–H groups and glycoside oxygen bridges. The exterior of γ-CD is relatively hydrophilic due to the primary hydroxyl groups located on carbon-6 and the secondary hydroxyl groups on carbons 2 and 3. As can be seen from Table 3, no CICS occurred for C_5, which is located inside of the cavity close to its narrow end [35,36]. But CICS was observed for all other carbons located on both the outside and inside (close to wider edge) of the cavity. These results suggested that compound A does not penetrate deeply into the γ-CD.

3.6. Proposal of complex structure

The proposed structure of the complex between compound A and γ-CD is shown in Fig. 9. The

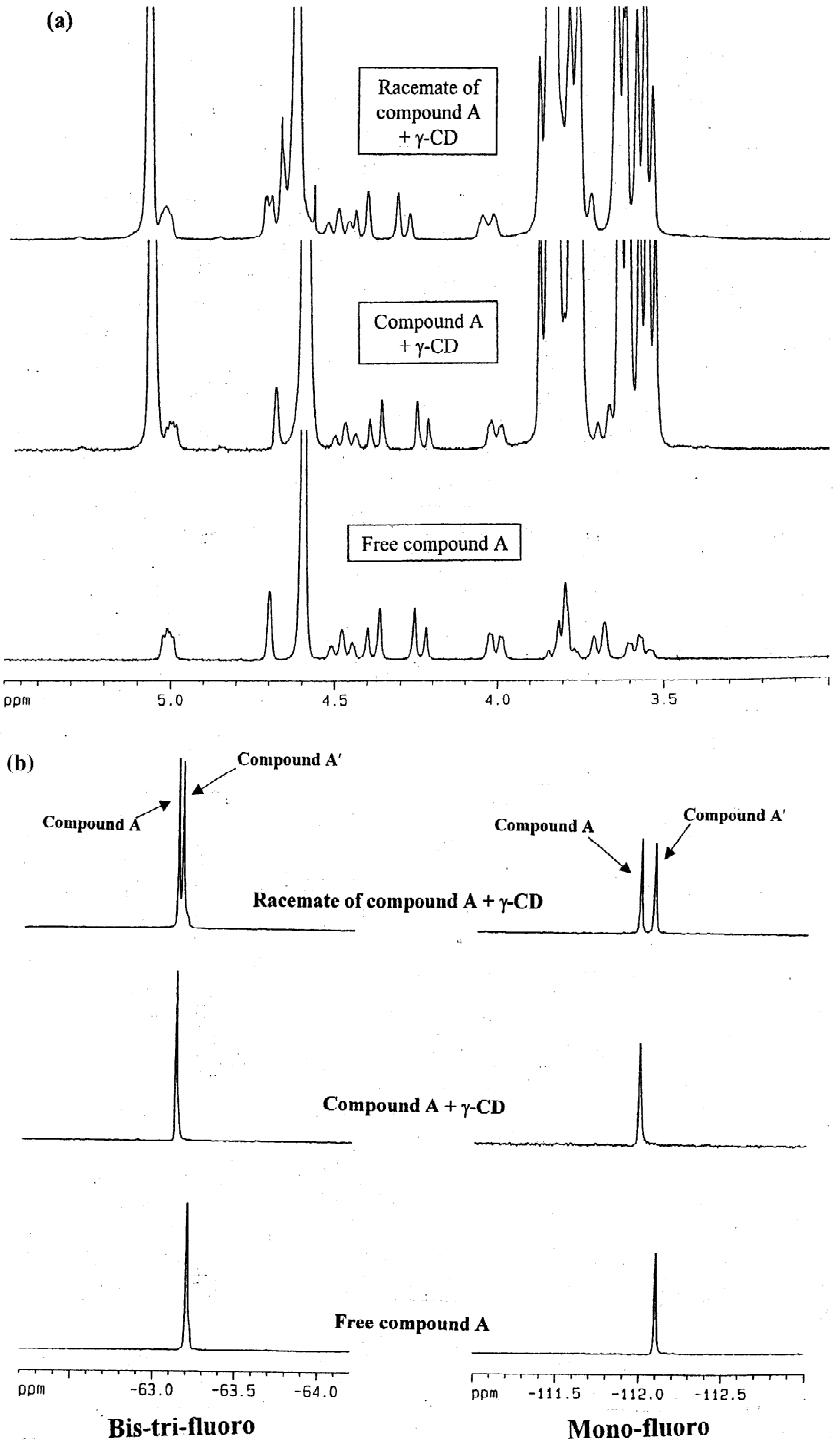

Fig. 8. A comparison of ^{1}H NMR spectra (a) and a comparison of ^{19}F NMR spectra (b). Conditions: the diluent for all sample solutions was acetonitrile-d_3–0.1% of formic acid-d_2 at a 20:80 (v/v) ratio. The concentration of both guest compound (compound A or its racemic mixture) and host (γ-CD) were 1.1×10^{-2} *M*.

Table 3
Carbon-13 complexation-induced chemical shift (CICS) differences ($\Delta\delta_{CICS}$) of γ-CD with compound A

Carbon position of γ-CD	δ (ppm) of free γ-CD	$\Delta\delta_{CICS}$ ($=\delta_{CICS}-\delta$)
C_1	102.2	−0.1
C_2	73.3	−0.1
C_3	72.6	−0.1
C_4	81.0	−0.1
C_5	72.1	0.0
C_6	60.4	−0.1

The conditions are the same as in Fig. 8.

molecule is partially included into the cavity with a 1:1 stoichiometry. The orientation of the complex structure with the inclusion of guest compound A is as follows: the mono-fluoro and bis-tris-fluoro phenyl rings are in the γ-CD cavity from the 2,3-dihydroxyl side, while the triazole and morpholine moieties stick out from cavity. These two moieties interact with hydroxyl groups at the wider end of the cavity through hydrogen bonding.

4. Conclusion

Chiral recognition with γ-CD can be evaluated by different analytical tools. The CE results demonstrated a direct view of enantioseparations. The elution order of the separation by CE and the binding constants that were determined by various techniques indicated that compound A interacts with γ-CD more strongly than its enantiomer. The existence of a molecular ion of the complex provides direct evidence of a 1:1 stoichiometric ratio. Complexation-induced chemical shifts (CICS) of compound A in proton NMR indicate that the complexation occurs upon addition of γ-CD and reveals possible hydrogen bonding between the morpholine ring and the hydroxy group on γ-CD. ^{19}F NMR provides direct evidence of the enantio-specific interactions between aromatic rings of the guest molecule and the γ-CD. ^{13}C NMR indicates that the guest compound has not penetrated deeply into γ-CD since no CICS of C_5 on the γ-CD was observed.

Among all the techniques used, NMR is superior in terms of structural elucidation, ESI-MS is superior in terms of providing direct evidence of the stoichiometric ratio, and CE is superior in terms of direct enantioseparation.

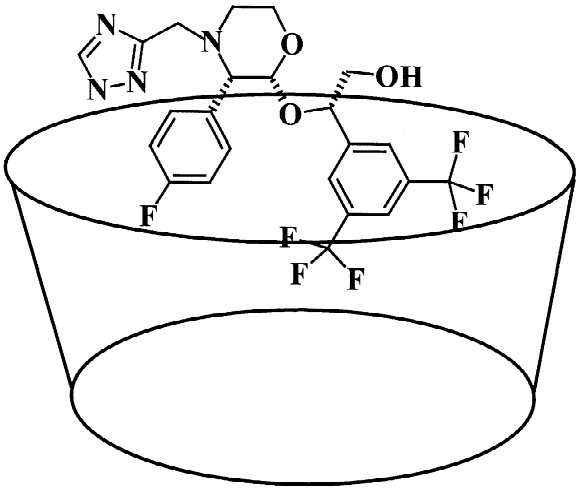

Fig. 9. A proposed complex structure.

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References

[1] B. Chankvetadze, G. Blaschke, J. Chromatogr. A 906 (2001) 309.
[2] N. Maier, P. Franco, W. Lindner, J. Chromatogr. A 906 (2001) 3.
[3] C. Blaschke, B. Chankvetadze, J. Chromatogr. A 875 (2000) 3.
[4] B. Chankvetadze, J. Chromatogr. A 792 (1997) 269.
[5] S. Fanali, J. Chromatogr. A 875 (2000) 89.
[6] K. Otsuka, S. Terabe, J. Chromatogr. A 875 (2000) 163.
[7] G. Subramanian, in: A Practical Approach to Chiral Separations by Liquid Chromatography, VCH, Weinheim, New York, 1994.
[8] K. Flohr, R.M. Paton, E.T. Kaiser, J. Am. Chem. Soc. 97 (1975) 1209.
[9] J. Martinie, J. Michon, A. Rassat, J. Am. Chem. Soc. 97 (1975) 1818.
[10] B. Chankvetadze, G. Schulte, G. Bergenthal, G. Blaschke, J. Chromatogr. A 798 (1998) 315.
[11] B. Chankvetadze, G. Schulte, G. Blaschke, J. Chromatogr. A 732 (1996) 183.

[12] G. Endresz, B. Chankvetadze, D. Bergenthal, G. Blaschke, J. Chromatogr. A 732 (1996) 133.
[13] B. Chankvetadze, G. Pintore, N. Burjanadze, D. Bergenthal, K. Bergander, J. Breitkreuz, C. Muhlenbrok, G. Blaschke, J. Chromatogr. A 875 (2000) 455.
[14] Y. Inoue, H. Hoshi, M. Sakurai, R. Chujo, J. Am. Chem. Soc. 107 (1985) 2319.
[15] S. Li, W.C. Purdy, Anal. Chem. 64 (1992) 1405.
[16] S.R. Gratz, A.M. Stalcup, Anal. Chem. 70 (1998) 5166.
[17] H. Shimizu, A. Kaito, M. Otagiri, Chem. Pharm. Bull. 23 (1975) 201.
[18] M. Blanco, J. Coello, H. Iturriaga, S. Maspoch, C. Perez-Maseda, Anal. Chim. Acta 407 (2000) 233.
[19] K. Harata, H. Uedaira, Bull. Chem. Soc. Jpn. 48 (1975) 375.
[20] E.C. Kempen, J.S. Brodbelt, Anal. Chem. 72 (2000) 5411.
[21] D.M. Hercules, J. Mass Spectrom. 36 (2001) 834.
[22] D.W. Armstrong, F.N. Spino, T.D. Golden, J. Am. Chem. Soc. 108 (1986) 1418.
[23] S.A. C Wren, R.C. Row, J. Chromatogr. 603 (1992) 235.
[24] S.A. C Wren, R.C. Row, J. Chromatogr. 609 (1992) 363.
[25] S.G. Penn, E.T. Bergstrom, D.M. Goodall, Anal. Chem. 66 (1994) 2866.
[26] Y.Y. Rawjee, R.L. Williams, G. Vigh, J. Chromatogr. A 652 (1993) 233.
[27] L. Zhou, J. Trubig, A. Dovletoglou, D.C. Locke, J. Chromatogr. A 773 (1997) 311.
[28] L. Zhou, R. Thompson, D. Ellison, D. Locke, J. Capillary Electrophor. 6 (1997) 279.
[29] L. Zhou, D.B. Johnson, C. Miller, J.M. Wyvratt, J. Chromatogr. A 875 (2000) 389.
[30] L. Zhou, R. Thompson, S. Song, D. Ellison, J.M. Wyvratt, J. Pharm. Biomed. Anal. 27 (2002) 541.
[31] R. Kuhn, F. Erni, Anal. Chem. 64 (1992) 2815.
[32] R.M. Mohseni, R.J. Hurtubise, J. Chromatogr. 499 (1990) 395.
[33] C. Moeder, T.O. Brien, R. Thompson, G. Bicker, J. Chromatogr. A 736 (1997) 1.
[34] N. Harada, in: Circular Dichroic Spectroscopy, Exiton Coupling in Organic Stereochemistry, University Books, VCH, New York, 1983.
[35] J. Szejtli, in: Cyclodextrins and Their Inclusion Complexes, Akademiai Kiado, Budapest, 1994.
[36] R.J. Bergeron, M.P. Meeley, Biorg. Chem. 5 (1976) 197.