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Evaluation of the interaction between β-cyclodextrin and psychotropic drugs by surface plasmon resonance assay with a Biacore® system

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ABSTRACT

Phase-solubility studies have been used to evaluate the solubilizing effects of cyclodextrins (CDs) on lipophilic, water-insoluble drugs. However, large amounts of CDs and drugs are required to measure solubility by phase-solubility studies. Thus, more efficient approaches to evaluate the interaction of CDs with drugs are needed. Herein we introduce a method that evaluates the interaction between immobilized β-cyclodextrin and psychotropic drugs by surface plasmon resonance assay with a Biacore® system. Association constants and stoichiometries observed were generally consistent with values calculated by traditional methods, such as phase-solubility and continuous variation methods. Results showed that the analytical method using Biacore® was suitable to evaluate CD–drug interactions.

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of 6, 7, or 8 D-glucose units, which are called α-, β-, and γ-CD, respectively. CDs form water-soluble inclusion complexes with many water-insoluble drugs. Thus, CDs are used as pharmaceutical excipients to improve water solubility. Furthermore, some recent studies have shown that CDs also form non-inclusion complexes as well as complex aggregates and complexes formed by an interaction with outside of CD [1–6]. Phase-solubility studies have been used to evaluate the solubilizing effects of CDs on lipophilic, water-insoluble drugs. However, several hundred milligrams of CDs are required to measure the solubility by phase-solubility studies on each drug. It may be difficult to evaluate the abilities of CDs to form non-inclusion complexes on the basis of phase-solubility studies. Furthermore, an evaluation of the abilities of large-ring CDs (LR-CDs) composed of more than 9 D-glucose units in the formation of inclusion complexes has been performed rarely due to low yields. Therefore, more efficient approaches are needed to evaluate the solubilizing effects of LR-CDs on various compounds.

A surface plasmon resonance (SPR) assay has successfully been used for the evaluation of molecular interactions [7]. The SPR assay allows detection of molecular interactions by monitoring changes in refractive index at gold surfaces on which ligands are immobilized. As compared with phase-solubility studies, the SPR assay has the advantage that CDs used as immobilized ligands are needed in only relatively small amounts, and the immobilized ligands are stable and useful for repeated measurements. The SPR assay has been utilized for determining association constants for non-covalent interactions of CDs with low molecular weight guests [8–10].

The first analytical instrument to use SPR technology was Biacore®, which came on the market in 1989. It uses a flow system to bring analyte solutions containing one binding compound into contact with a gold surface onto which the second binding compound is covalently immobilized. The SPR signal, or response, is measured in resonance units (RU) and can be monitored in real-time, allowing the determination of binding data, such as the association constant, dissociation constant, reaction rate constant and stoichiometry.

To establish a method for estimating the abilities of CDs to form inclusion complexes using small amounts of compounds and short experiment times, we investigated the possibility of a screening system using a Biacore® instrument for the evaluation of interactions between CDs and various drugs. We studied non-covalent interactions between a conventional β-CD and psychotropic drugs as models by SPR assay with a Biacore® system. The results obtained by Biacore® were compared with the kinetic data calculated by conventional methods, such as phase-solubility and continuous variation methods.

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2. Experimental

2.1. Reagents and chemicals

β-CD was obtained from Nihon Shokuhin Kako Co. (Tokyo, Japan). Biacore®-specific products, such as the Sensor Chip CM5 (research grade), HBS-N (0.01 M HEPES pH 7.4, 0.15 M NaCl) and chemicals required for covalent immobilization, were purchased from GE Healthcare UK Ltd. (Buckinghamshire, England). Other chemicals were analytical grade reagents from commercial sources without further purification. Milli-Q Water (Milli-Q Gradient, Millipore Co., USA) was used in all experiments as ultrapure water.

2.2. Preparation of 6-deoxy-6-amino-β-cyclodextrin

First, β-CD was modified to 6-deoxy-6-tosyl-β-CD (β-CD-OTs) by *p*-toluenesulfonyl chloride in pyridine at 25 °C, as described in the literature [11,12]. Then, 6-deoxy-6-amino-β-CD (β-CD-NH_2) was prepared by amination of β-CD-OTs according to a slightly modified method reported by Petter et al. [13]. Synthesized β-CD-OTs and β-CD-NH_2 were identified by MALDI-TOF MS and NMR. Yield from β-CD to β-CD-NH_2 was 5.3%.

2.3. Immobilization of β-CD-NH_2

The work described in this paper was performed on a Biacore® 2000 (GE Healthcare UK Ltd., Buckinghamshire, England) with HBS-N used as continuous flow buffer. All experiments using Biacore® were carried out at 25 °C. For immobilization of β-CD-NH_2, the flow rate was 5 μL/min. The carboxymethyl dextran on the surface of the sensor chip was activated by injecting 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 0.39 M) and *N*-hydroxysuccinimide (NHS, 0.04 M) onto the sensor chip surface for 10 min (Fig. 1, Step 1). The β-CD-NH_2 was dissolved in buffer containing 10 mM disodium tetraborate and 1.0 M NaCl at pH 8.5. The solution was filtered and injected onto the sensor chip surface to allow β-CD-NH_2 to react for 10 min (Fig. 1, Step 2). Any remaining ester groups on the sensor chip were inactivated by injection of 1 M ethanolamine at pH 8.5 for 7 min (Fig. 1, Step 3). One Biacore® sensor chip contains four separated surfaces called flow cells 1–4 (Fc. 1–4). Moreover, one of the surfaces is required as a reference cell in a typical experiment. As a reference cell, Fc. 1 was activated using EDC/NHS and blocked with ethanolamine in the same way as immobilization of β-CD-NH_2.

2.4. Detections of the specific binding to β-CD

In this experiment, sertraline hydrochloride (STL), perospirone hydrochloride hydrate (PSR), risperidone (RPD), quetiapine fumarate (QTP), trazodone hydrochloride (TZD) and amoxapine (AXP) were used because the drugs were expected to interact with β-CD due to molecular structures containing benzene rings (Fig. 2). Each drug was dissolved in HBS-N at 100 μM concentration. Sample solutions prepared in this way were injected into the flow system at 20 μL/min for 2 min, and then changes in response were monitored. For each drug, the response differences (Resp. Diff.) between Fc. 1 and the flow cell with immobilized β-CD (Im-β-CD) on the steady state were determined.

2.5. Calculation of association and dissociation constants, and rate constants of association and dissociation

For the drugs for which the specific binding to Im-β-CD was detected, five concentrations were prepared by serial dilution with HBS-N. The concentrations for each sample solution were as follows: 6.25, 12.5, 25, 50 and 100 μM for RPD, QTP and AXP, 31.25, 62.5, 125, 250 and 500 μM for PSR, and 62.5, 125, 250, 500 and 1000 μM for STL and TZD. These five concentration solutions and HBS-N were injected into the flow system at 20 μL/min for 2 min, and then the changes in response were monitored. Once a drug sample was injected into the flow system, the association phase was monitored for 120 s, and then the dissociation phase was monitored for 150 s. After the observation of the dissociation phase, 20% ethanol was injected for 30 s to regenerate the surface and next measurement was carried out following attainment of equilibrium by HBS-N. The responses at each concentration were measured in duplicate. The rate constants of association and dissociation were calculated by non-linear fitting method including the simultaneous fitting for the association and dissociation phases in each sensorgram in BIAevaluation software version 4.1 (GE Healthcare UK Ltd., Buckinghamshire, England).

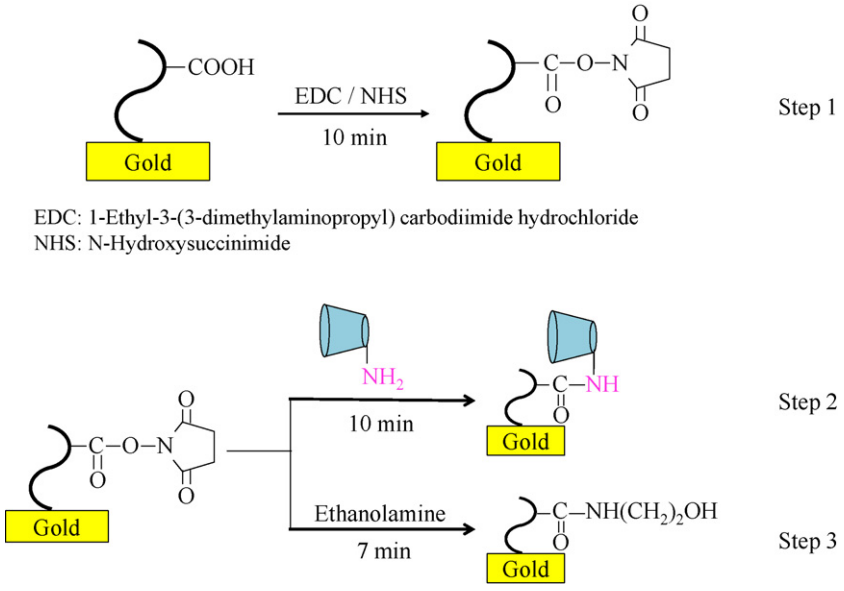
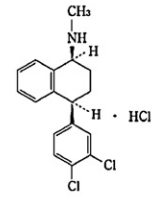
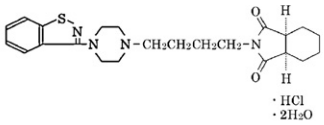
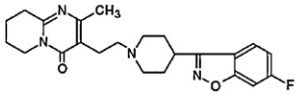
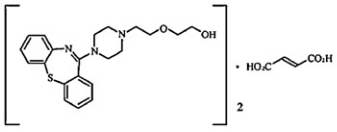
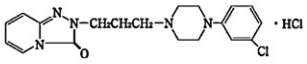
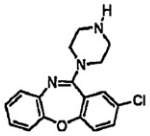


Fig. 1. Immobilization of β-CD-NH_2.

	Chemical structure	Molecular weight	pK_a
Sertraline hydrochloride (STL)		342.69	9.5
Perospirone hydrochloride hydrate (PSR)		499.07	7.2
Risperidone (RPD)		410.48	8.2
Quetiapine fumarate (QTP)		883.09	6.8
Trazodone hydrochloride (TZD)		408.32	6.7
Amoxapine (AXP)		313.78	7.6

The molecular weight and pKa values except for amoxapine came from data published by production company. Each value of amoxapine were quoted from 13th Merck index.

Fig. 2. Structural formulae, molecular weights and acid dissociation constants of six psychotropic drugs.

2.6. Phase-solubility studies

Solubility experiments for AXP and TZD were carried out according to the methods of Higuchi and Connors [14]. Excess amounts of each drug were added to HBS-N solutions containing various concentrations of β-CD, and the mixtures were shaken at 25 °C for 7 days. After reaching equilibrium, these sample solutions were filtered using a membrane filter (0.45 μm). Each sample solution was diluted at appropriate concentrations by HBS-N and analyzed by spectrophotometer at the following wavelengths: AXP at 296 nm and TZD at 247 nm. All absorbance measurements were performed on a UV/VIS spectrophotometer V-560 (Jasco Co., Ltd., Tokyo, Japan).

The association constant (K_A) was calculated for each drug using the slope and the intercept (S_0) obtained from the initial upward linear portion of the phase-solubility diagram and using the following equation:

$$K_A = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

The stoichiometry of the inclusion complex can be calculated by analysis the length of plateau region according to the following equation, if the phase-solubility diagram shows B_S type having plateau region.

$$\frac{[\text{Guest}]}{[\text{CD}]} = \frac{[\text{Total guest added to system}] - [\text{Guest in solution at the plateau region}]}{[\text{CD corresponding to plateau region}]} \quad (2)$$

In case of TZD, the phase-solubility diagram showed B_S type and the stoichiometry was 1:2 (TZD:β-CD).

2.7. Continuous variation method

This experiment for AXP was carried out according to the methods of Job [15], because the solubility-phase diagram of AXP showed A_L type and the stoichiometry was not determinable. Original solutions of AXP and β-CD were prepared at 5.0×10^{-4} M in HBS-N. The complex AXP/β-CD was formed at a constant volume by adding various volume ratios of AXP solution and β-CD solution at several molar ratios $r = [\text{AXP}]/([\text{AXP}] + [\beta\text{-CD}])$, varying from 0.1 to 0.9 to reach a final total molarity of 2.5×10^{-6} M. The mixtures were shaken at 25 °C for 2 days and then were filtered using a membrane filter (0.45 μm). The sample solution was diluted at appropriate concentrations by HBS-N and analyzed by spectrophotometer at 296 nm. The absorbance difference $\Delta A = A_0 - A$ was determined by measuring the absorbance of AXP with β-CD (A) and without β-CD (A_0). The product $\Delta A \times [\text{AXP}]$ versus r was then plotted to determine the stoichiometry of the com-

plex, which was 1:1 when $\Delta A \times$ [AXP] reached its maximum for $r = 0.5$.

3. Results and discussion

3.1. Immobilization of β-CD-NH_2 and detection of the specific binding to β-CD

The immobilized level was 505.8 RU, which showed that the occupancy of Im-β-CD on the surface of sensor chip was 26.8 molecules/100 nm^2. Because it may be difficult to form complex aggregates due to the inhibition of β-CD mobility by immobilization of β-CD and the low occupancy of Im-β-CD, we expected the possibility that the method using Biacore® selectively evaluated the abilities of CDs in forming inclusion complexes with several drugs.

As shown in Fig. 3, Resp. Diff. in the range 3.8–9.4 RU were observed for each psychotropic drug. It was suggested then that each psychotropic drug formed specific binding to Im-β-CD.

3.2. Calculation of association and dissociation constants, and rate constants of association and dissociation

Fig. 4 shows the binding patterns of each psychotropic drug to Im-β-CD. For each psychotropic drug, except RPD, the sensorgram was the general curve observed by Biacore®. As shown in Fig. 4c, the sensorgram of RPD was the curve of a box shape, suggesting that the association rate and the dissociation rate were very fast. Kinetic analysis of RPD was difficult to perform because the slope of the curve could not be obtained due to the short association phase and dissociation phase. Therefore, an affinity analysis on the basis of Langmuir type adsorption equilibrium was applied because an equilibrium of inclusion complex formation was quickly reached.

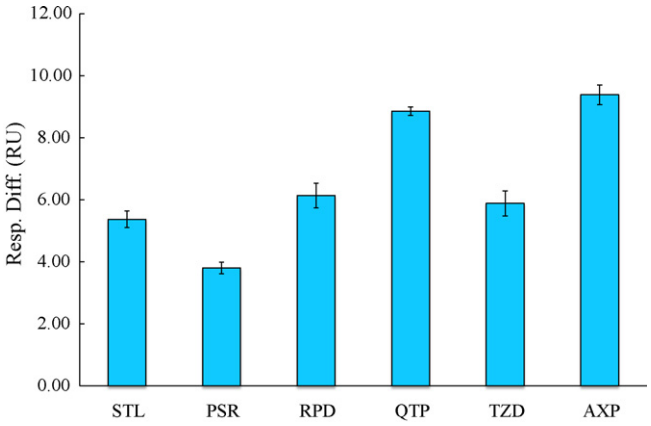


Fig. 3. Interactions between β-CD and six psychotropic drugs. Each value represents the mean ± SD of three measurements.

For affinity analysis, the K_A value was calculated using the following equation:

$$R_{eq} = \frac{K_A \cdot C \cdot R_{max}}{1 + K_A \cdot C} \quad (3)$$

where R_{eq} is the response at equilibrium, C is the RPD concentration and R_{max} is the theoretical value of the response in association of RPD with all Im-β-CD molecules.

The stoichiometry (n) was also calculated using the following equation:

$$n(\text{Drug}/\beta\text{-CD}) = \frac{R_{max}/MW_{Drug}}{R_{Im}/MW_{\beta\text{-CD-NH}_2}} = \frac{R_{max} \times MW_{\beta\text{-CD-NH}_2}}{R_{Im} \times MW_{Drug}} \quad (4)$$

where $MW_{\beta\text{-CD-NH}_2}$ and MW_{Drug} are the molecular weights of β-CD-NH_2 and drug, and R_{Im} is the immobilized level of β-CD-NH_2.

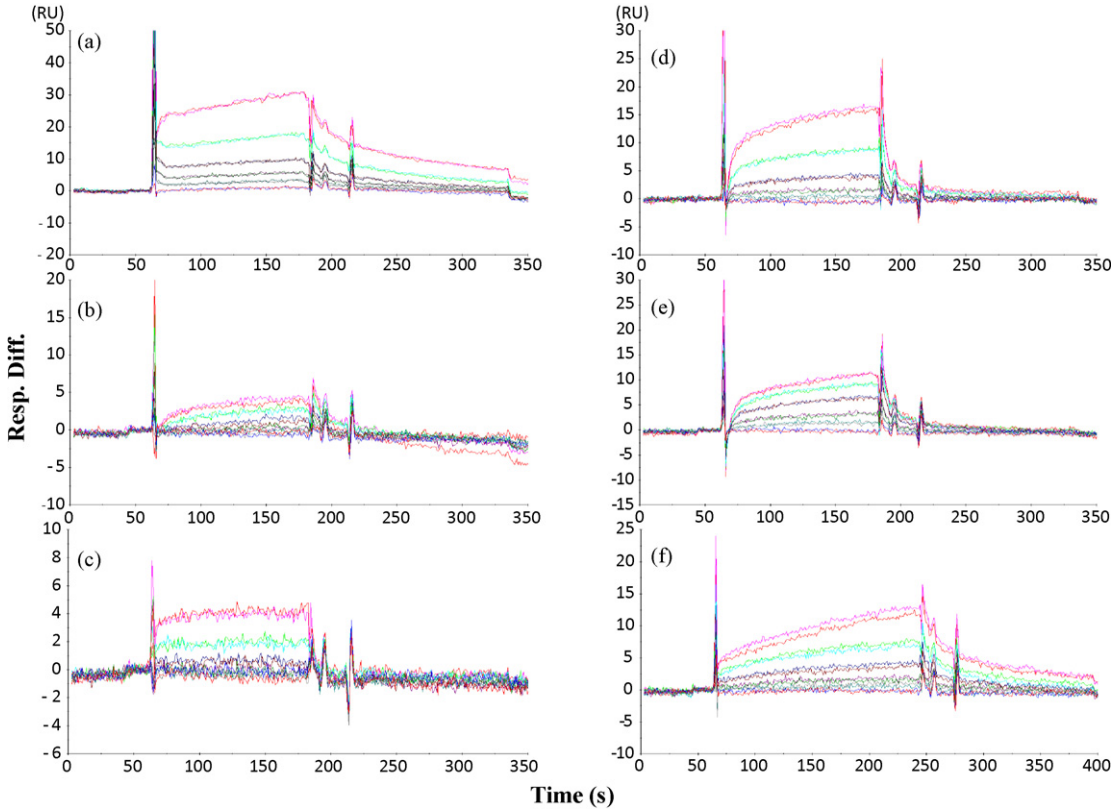


Fig. 4. Sensorgrams of (a) STL, (b) PSR, (c) RPD, (d) QTP, (e) TZD and (f) AXP.

Table 1
Kinetic parameters of psychotropic drugs with Im-β-CD.

Drug	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_A (M^{-1})	K_D (M)	R_{max} (RU)	n
STL	2.17	6.08×10^{-3}	345	3.67×10^{-3}	126.9	0.83
PSR	k_{a1}: 20.2 k_{a2}: 1.26×10^{-4}	k_{d1}: 0.111 k_{d2}: 5.69×10^{-3}	182	5.50×10^{-3}	80.7	0.42
RPD	–	–	309	3.74×10^{-3}	167.0	0.91
QTP	54.4	4.34×10^{-2}	1129	3.85×10^{-4}	164.6	0.96
TZD	k_{a1}: 10.4 k_{a2}: 9.56×10^{-4}	k_{d1}: 8.28×10^{-2} k_{d2}: 4.45×10^{-2}	125	8.02×10^{-3}	73.0	0.44
AXP	8.53	1.51×10^{-2}	586	1.77×10^{-3}	163.0	1.16

k_a: association rate constant; k_d: dissociation rate constant; K_A: association constant; K_D: dissociation constant; n: stoichiometry (drug/Im-β-CD).

The values of R_{max} and R_{Im} are directly proportional to masses of drug and β-CD-NH_2, respectively. The association parameters obtained are summarized in Table 1. The association constants of psychotropic drugs were 10^2–10^3 M^{-1} levels for Im-β-CD. QTP had a relatively high affinity of 1129 M^{-1} with Im-β-CD. Association rate constant (k_a) and dissociation rate constant (k_d) are parameters that express the formation rate of complex and the stability of complex, respectively. The k_a and k_d values of STL, QTP and AXP were evaluated. The k_d value of QTP was the highest value of the three drugs, which suggests that the dissociation of QTP from Im-β-CD was relatively fast. The k_a value of QTP was 6–25 times higher than the k_a values of STL and AXP, although little difference among the k_d values was observed. This was considered to mean that the fast formation rate of complex reflected the high affinity of QTP for Im-β-CD. It has been reported that some CDs represent an induced-fit model of binding rather than one following the rigid lock-and-key type in inclusion complexation with guest molecules [16–18]. Because one of the factors in the determination of k_a is a skewed binding conformation, we could consider the possibility that the cavity of Im-β-CD underwent conformational changes in order to form inclusion complexes with QTP. The comparison of k_a and k_d between STL and AXP suggests that STL bound to Im-β-CD slowly and formed a relatively stable complex. It was considered that the stability of the inclusion complex of Im-β-CD with STL was affected by hydrogen bonds and van der Waals forces which were the primary factors in determination of k_d. Results calculated using Eq. (4) demonstrated that the complexes of STL, RPD, QTP and AXP with Im-β-CD had 1:1 (drug: Im-β-CD) stoichiometries and the complexes of PSR and TZD with Im-β-CD had 1:2 stoichiometries.

A commonly used measure for accuracy of the fitted parameter is χ^2, which describes the residual noise per data point after fit, according to the following equation:

$$\chi^2 = \frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n - p} \quad (5)$$

where $\hat{y}_i$ is the estimate of the measured data point y_i, n is the number of data points, and p is the number of parameters fitted by the optimizer. It is described in BIAevaluation Software Handbook version 3 that values of χ^2 below 10 are acceptable. The range of χ^2 values for each psychotropic drug, except STL, was 0.12–3.05, which suggested that the fits were acceptable. Although the χ^2 value of STL was 17.95, the fit could still be considered acceptable due to the shape of the residual plot.

3.3. Phase-solubility studies and continuous variation method

It has been known that aminated β-CDs are positively charged through the protonation at pH 7.2 [19]. As the result, the binding ability enhances toward negatively charged guest molecules through additional electrostatic interactions in the opposite charged host–guest complexation. However, the six drugs used in this study do not have a negative charge in the molecule and amino group introduced to β-CD was not free due to the linker to carboxymethyl dextran on the sensor chip. From these reasons, we expected that the effect of the introduction of amino group to β-CD was not strong on inclusion complex formation in our study. Thus, β-CD instead of aminated β-CD was used in the phase-solubility studies and continuous variation method.

3.3.1. Amoxapine

As shown in Fig. 5a, the phase-solubility diagram of AXP and β-CD was A_L type. The K_A value determined from the initial slope was 379 M^{-1}, which was comparable to the K_A value (586 M^{-1}) obtained by SPR assay with Biacore®.

3.3.2. Trazodone

As shown in Fig. 5b, the phase-solubility diagram of TZD and β-CD was the B_S type. The K_A value determined from the initial slope was 122 M^{-1}, which was essentially equivalent with the K_A value (125 M^{-1}) obtained by SPR assay with Biacore®. The stoichiometry of the complex was then analyzed using the plateau portion of the phase-solubility diagram and was found to be 1:2 (TZD:β-CD), which agreed with the stoichiometry calculated by SPR assay with Biacore®.

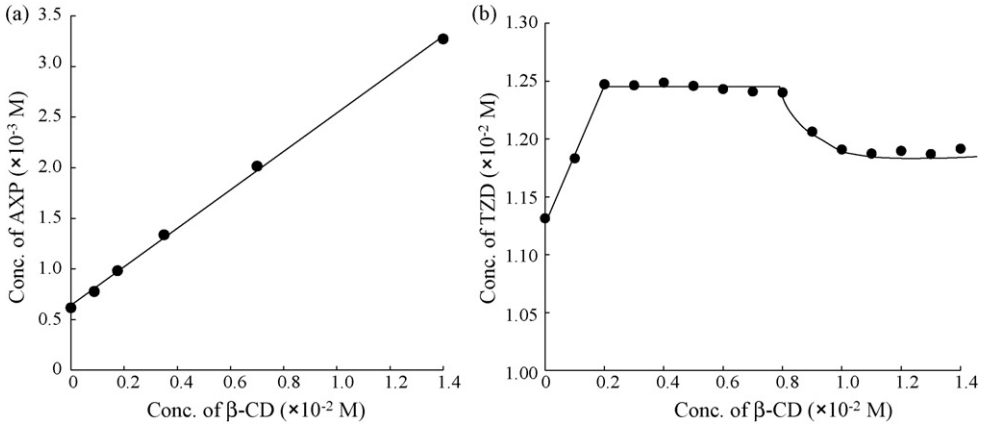


Fig. 5. Phase-solubility diagrams of (a) β-CD-AXP system and (b) β-CD-TZD system in HBS-N at 25 °C.

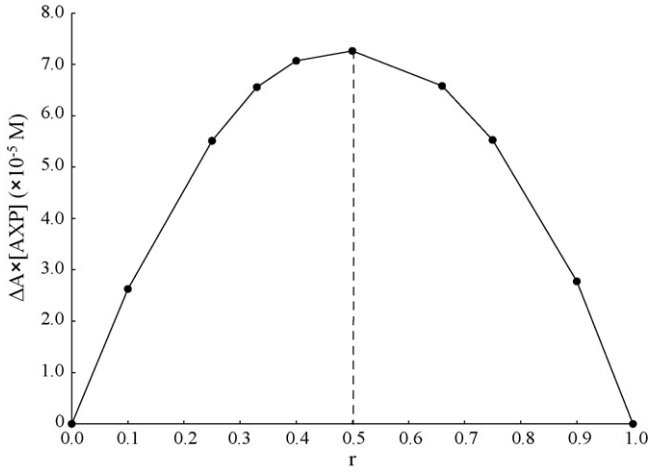


Fig. 6. Continuous variation plot (Job's plot) of β-CD-AXP system in HBS-N at 25 °C.

Table 2
The quantities of β-CD and drugs used in each evaluation method, and the experimental times of those methods.

	Biacore®	Phase-solubility method	Continuous variation method
β-CD	40 mg	300–1400 mg	20 mg
Drug	6–80 μg	30–750 mg	5 mg
Experimental time	30–120 min	3–8 days	3 day

Fig. 6 represents a Job's plot of $\Delta A \times$ [AXP] as a function of r = [AXP]/([AXP] + [β-CD]). The plot showed a maximum value at r = 0.5, corresponding to 1:1 stoichiometry. The stoichiometry agreed with the stoichiometry calculated by SPR assay with Biacore®.

The amounts of β-CD and drugs used in these studies and the experimental times are summarized in Table 2. As compared with conventional methods, SPR assay with a Biacore® system has the advantages that the required amounts of CDs and drugs are small and that the interaction between CDs and drugs can be evaluated rapidly and easily.

4. Conclusion

β-CD-NH_2 was successfully immobilized to the surface on the sensor chip. However, since the yield of β-CD-NH_2 was low, a more efficient approach to amination of CDs will be required to apply to LR-CDs.

The results of binding experiments using SPR assay with a Biacore® system indicated that the six psychotropic drugs formed specific binding to Im-β-CD. For AXP and TZD, the association constants and the stoichiometry obtained by Biacore® generally corresponded well with the kinetic values calculated by the conventional methods, such as phase-solubility method and continuous variation method. The results showed that SPR assay with a Biacore® system was useful to evaluate the interaction between CDs and various drugs. It was also possible that the SPR assay with a Biacore® system selectively evaluated the abilities of CDs in forming inclusion complexes, since the mobility of CDs was inhibited due to the immobilization of CDs to the surface on the sensor chip. Furthermore, the evaluation of interactions between CDs and drugs on the basis of SPR assay with a Biacore® system has advantages on the required amounts of CDs and drugs and the time for measurements in comparison with the conventional methods such as the phase-solubility study and the continuous variation method.

Acknowledgments

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