A Study of 1:1 Plus 1:2 Complexes between Barbiturate and α-Cyclodextrin Using the Freezing Point Depression Method[1,2]

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The freezing point depression method for studying drug interaction has been extended to a system containing both 1:1 (AB) and 1:2 (AB_2) complexes. The osmotic concentration of dilute aqueous solutions was measured by this method with a commercially available osmometer. On the basis of the colligative properties, a mathematical model has been proposed to calculate the apparent stability constants (K_1 and K_2). This method is applied to complexes of α-cyclodextrin with three barbiturate derivatives (barbital, phenobarbital, and pentobarbital). The results showed that the apparent stability constants obtained were in fair agreement with those obtained by the spectroscopic method. The advantage of this method is that the apparent stability constant could be estimated quickly using a simple procedure.

Keywords freezing point depression; 1:2 complex; stability constant; osmotic pressure; inclusion complex; barbiturate

Several methods for determining the stability constants of complex systems containing both 1:1 and 1:2 stoichiometric ratios have been reported using a variety of techniques, such as solubility,[3] kinetic,[4] and spectroscopic inhibitor methods.[5] These methods, however, do not appear to be suitable for systems that involve no spectral changes, and it takes them a long time to perform their operations.

In our previous paper, cyclodextrin complexes were successfully applied to rapid and accurate analyses of various alcohols by the freezing point depression method.[2] The application of this method, however, has been limited to the study of 1:1 complex systems.

In the present study, the freezing point depression method was extended to complex systems containing both 1:1 and 1:2 stoichiometric ratios, such as barbiturate/α-cyclodextrin (α-CD) systems. These interactions are known to form 1:2 complexes, as previously measured by the solubility method or the spectroscopic inhibitor method. We therefore examined the applicability of the freezing point depression method for 1:1 plus 1:2 complex systems by comparing the stability constants obtained by the freezing point depression method with those values obtained by the spectroscopic method. This paper describes the theory and practice of the freezing point depression method that permits its application in systems containing both 1:1 and 1:2 complexes.

Experimental

Materials α-CD, obtained from Nihon Shokuhin Kako Co., Ltd., was recrystallized twice from hot water and dried for 3 h at 110 °C. Reagent grade barbital sodium, phenobarbital sodium, and pentobarbital sodium, obtained from Tokyo Kasei Kogyou Co., Ltd., were used after drying without purification. Distilled de-ionized water was used.

Apparatus Osmotic measurements were made using an osmometer (Osmette Model 2007, Precision Systems., Inc.), which was calibrated with standard solutions (100 mOsm/kg, 500 mOsm/kg) of dextrose supplied by the company. The instrument was built according to principles and practices previously described.[2]

Measurement of Osmotic Concentration Solutions were prepared with distilled water in such a way that each final concentration was 50 mM in α-CD, in which the concentration of the barbiturate derivatives varied from 0 to 100 mM. In all runs, the pH values were observed to be constant, respectively, at 10.0, 8.7, and 9.9 for barbital/α-CD system, phenobarbital/α-CD system, and pentobarbital/α-CD system. Osmotic concentrations were measured using 2 ml of each sample and were replicated three times for each solution. The reproducibility of the measurement was reported previously; for the 50 mM solution, it can be within 1%.

Spectroscopic Measurement The UV absorption was made on a Hitachi 320 spectrometer at either 10 °C, 20 °C, or 30 °C in a temperature-controlled cell. Each solution, at variable α-CD concentration such that the final concentration ranged from 0.5 mM to 20 mM, was prepared so that the barbiturate concentration was maintained at 0.1 mM. The reference solution was prepared with the same concentration of α-CD. Absorbance was measured, respectively, at 254, 256, and 252 nm for barbital/α-CD system, phenobarbital/α-CD systems, and pentobarbital/α-CD system. The stability constants were calculated as previously described.[6]

Theoretical

For a solution with only one solute dissolved, the osmotic concentration ($\bar{m}$) of an aqueous solution can be defined as $\bar{m}=\nu\cdot\phi\cdot m$, where ν represents the number of ions formed by solvolysis from a solute, $\nu=1$ the inionized solutes, ϕ the molal osmotic coefficient, and m the molality of the solute. In a dilute solution, the osmotic concentration is proportional to the molarity of the solution, that is, $\phi=1$, and molality is equal to molarity.[7]

Let us assume that the interaction process takes place *via* two-step equilibria, where A represents the substrate and B represents the ligand. The apparent stability constants, K_1 and K_2, are given by Eq. 1 and Eq. 2, respectively.

$$A+B=AB \qquad K_1=[AB]/[A]\cdot[B] \tag{1}$$

$$AB+B=AB_2 \quad K_2=[AB_2]/[AB]\cdot[B] \tag{2}$$

For a mixture containing two solutes, the total molar concentration, M, and osmotic concentration, $\bar{M}$, can be defined by Eq. 3 and Eq. 4.

$$M=[A]+[B]+2[AB]+3[AB_2] \tag{3}$$

$$\bar{M}=[A]+[B]+[AB]+[AB_2] \tag{4}$$

The (known) total concentration of solute A and solute B are:

$$A_0=[A]+[AB]+[AB_2] \tag{5}$$

$$B_0=[B]+[AB]+2[AB_2] \tag{6}$$

The average number ratio ($\bar{r}$) of solute B bound per solute

A is:

$$r=\frac{B_0-[B]}{A_0}=\frac{[AB]+2[AB_2]}{[A]+[AB]+[AB_2]} \quad (7)$$

Substitution of Eq. 1 and Eq. 2 into Eq. 7 gives:

$$r=\frac{M-\bar{M}}{A_0}=\frac{K_1[B]+2K_1K_2[B]^2}{1+K_1[B]+K_1K_2[B]^2} \quad (8)$$

[B] is deduced from Eq. 4 and Eq. 5.

$$[B]=\bar{M}-A_0 \quad (9)$$

Substitution of Eq. 9 into Eq. 8 with rearrangement gives:

$$\frac{M-\bar{M}}{(\bar{M}-A_0)(\bar{M}-B_0)}=K_1+K_1K_2\frac{(\bar{M}+A_0-B_0)(\bar{M}-A_0)}{(\bar{M}-B_0)} \quad (10)$$

$$\frac{\Delta}{(A_0-\Delta)(B_0-\Delta)}=K_1+K_1K_2\frac{(2A_0-\Delta)(B_0-\Delta)}{(A_0-\Delta)} \quad (11)$$

Where Δ is the difference between M and $\bar{M}$, which can be obtained experimentally.

$$\Delta=M-\bar{M} \quad (12)$$

Therefore, the apparent stability constants, K_1 and K_2, can be obtained from the slopes and intercept of a linear plot, according to Eq. 11. If $K_2=0$, a 1 : 2 complex is not formed, and the slope of this line will be zero; thus Eq. 11 gives a simple plotting form: $K=\Delta/(A_0-\Delta)(B_0-\Delta)$.

Results

Complex Stoichiometry and Stability Constant Figure 1 shows the relationship between the experimentally determined osmotic concentration ($\bar{M}$) of the mixture and the sum of the respective concentrations (M) for barbital/α-CD, phenobarbital/α-CD, and pentobarbital/α-CD systems. The difference between M and $\bar{M}$ can be considered as evidence of the complex formation of a barbiturate with α-CD. To elucidate the stoichiometric ratio, the ratio of $\bar{M}/M$ was plotted against the mole ratio of [barbiturate]/[α-CD], as shown in Fig. 2. A minimum osmotic coefficient was estimated at a barbiturate mole ratio of 0.5 by extrapola-

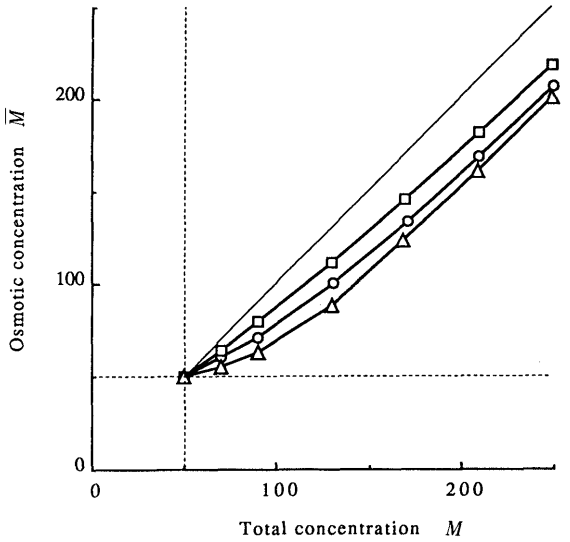

Fig. 1. Plots of Experimentally Determined Osmotic Concentration as a Function of the Total Molar Concentration for Aqueous Solutions of Barbital (○), Phenobarbital (□), and Pentobarbital (△)

α-CD concentration was 50 mM, and barbiturate concentration varied from 0 to 100 mM. A straight line represents $M=\bar{M}$.

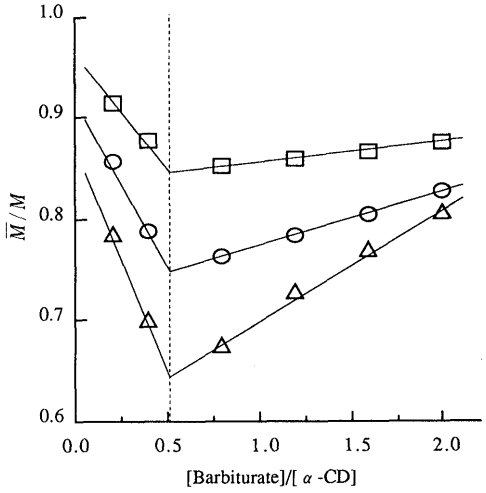

Fig. 2. The Ratio of $\bar{M}/M$ Changes of Barbiturate/α-CD System in Aqueous Solution

○, barbital/α-CD system; □, phenobarbital/α-CD system; △, pentobarbital/α-CD system. Total concentration of α-CD and barbiturate is 50 mM.

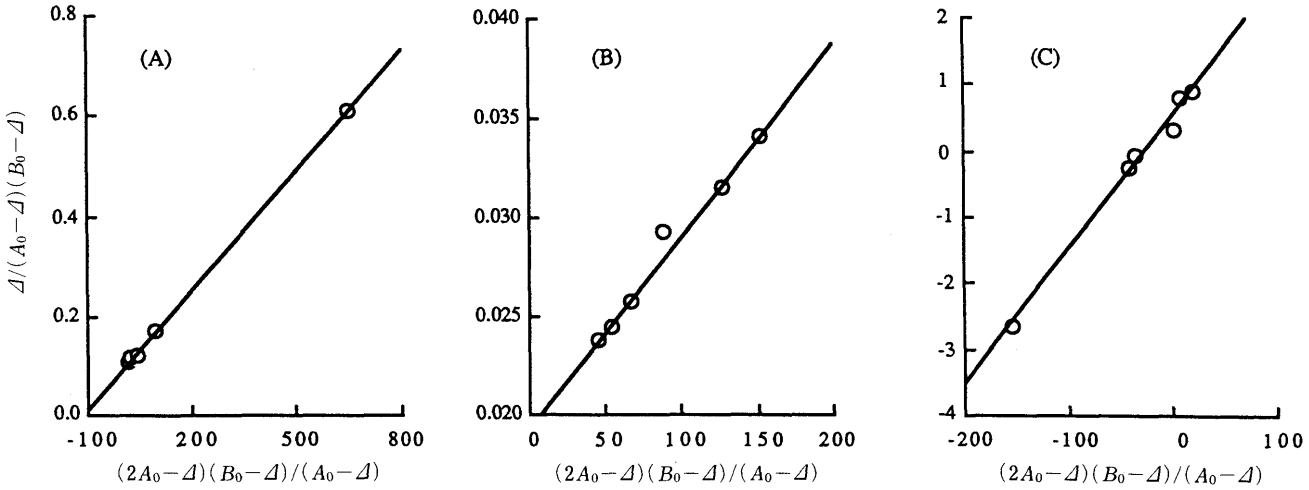

Fig. 3. Plots of Eq. 11 for the Barbiturate/α-CD System

A, barbital/α-CD system; B, phenobarbital/α-CD system; C, pentobarbital/α-CD system.

TABLE I. Experimentally Determined Osmotic Concentration and Calculated $\Delta/(A_0-\Delta)(B_0-\Delta)$ and $(2A_0-\Delta)(B_0-\Delta)/(A_0-\Delta)$ for Aqueous Solutions of α-CD/Barbital Na, α-CD/Phenobarbital Na and α-CD/Pentobarbital Na at Freezing Point

Compound (mM)		Osmotic concentration (mOsm/kg)	Δ (mOsm/kg)	$\frac{(2A_0-\Delta)(B_0-\Delta)}{(A_0-\Delta)}$ (mM)	$\frac{\Delta}{(A_0-\Delta)(B_0-\Delta)}$ (mM^{-1})
α-CD	50	50			
Barbital Na	10	20			
	20	40			
	40	81			
	60	121			
	80	160			
	100	200			
α-CD 50+barbital Na	10	60	10	—	—
50+	20	71	19	651	0.61
50+	40	100	31	100	0.17
50+	60	134	37	46	0.12
50+	80	169	41	27	0.12
50+	100	207	43	19	0.11
Phenobarbital Na	10	20			
	20	40			
	40	80			
	60	120			
	80	160			
	100	199			
α-CD 50+phenobarbital Na	10	64	6	154	3.4×10^{-2}
50+	20	79	11	126	3.1×10^{-2}
50+	40	111	19	90	2.9×10^{-2}
50+	60	146	24	69	2.6×10^{-2}
50+	80	182	28	56	2.4×10^{-2}
50+	100	218	31	47	2.4×10^{-2}
Pentobarbital Na	10	20			
	20	40			
	40	80			
	60	119			
	80	159			
	100	199			
α-CD 50+pentobarbital Na	10	55	15	−35	−0.086
50+	20	63	27	−43	−0.17
50+	40	88	42	−152	−2.6
50+	60	123	46	22	0.85
50+	80	161	48	7.1	0.76
50+	100	201	48	5.9	0.47

tion of the observed values. These results suggest that barbiturates form a 1 : 2 complex with α-CD; therefore, the analysis of these complex systems should be carried out as a system containing both 1 : 1 and 1 : 2 complexes.

Osmotic data were treated according to Eq. 11 (Table I), and the profiles of these data, plotted as $\Delta/(A_0-\Delta)(B_0-\Delta)$ *vs.* $(2A_0-\Delta)(B_0-\Delta)/(A_0-\Delta)$, are presented in Fig. 3. A linear relationship was observed. K_1 and K_2 were calculated from the slope and intercept of the linear plot and are shown in Table II. If a given drug interaction does not conform to a model described in Theoretical, the plot does not give a straight line, and if the straight line has no slope, slope = 0, the stoichiometric ratio of complex is 1 : 1, in a word, $K_2 = 0$.

Comparison with Spectroscopic Method To evaluate the reliability of the freezing point depression method, the stability constants obtained by this method were compared with those obtained by the spectroscopic method. For the freezing point depression method, the stability constant reveals the values to be around 0 °C, *i.e.*, the freezing point. Therefore, the stability constants determined at various temperatures (10 °C, 20 °C, or 30 °C) by the spectroscopic method were extrapolated to 0 °C according to van't Hoff plots.

TABLE II. Comparision of Stability Constants of Barbiturate and α-CD Complexes Determined by the Freezing Point Depression Method and the Spectroscopic Method

Compound	Method	Stability constant	
		K_1 (M^{-1})	K_2 (M^{-1})
Barbital	Freezing point depression	94± 9	9.5±2.1
	(Spectroscopic)	(84)	(9.2)
Phenobarbital	Freezing point depression	20± 1	4.9±0.3
	(Spectroscopic)	(20)	(6.7)
Pentobarbital	Freezing point depression	527±33	44 ±8
	(Spectroscopic)	(435)	(42)

Each value represents the mean ± S.D. of three determinations. ( ): The K values determined by the spectroscopic method are K values extrapolated to 0 °C.

The results are summarized in Table II and Fig. 4. The estimated K_1 and K_2 came very close to those by the freezing point depression method. It is apparent that the freezing

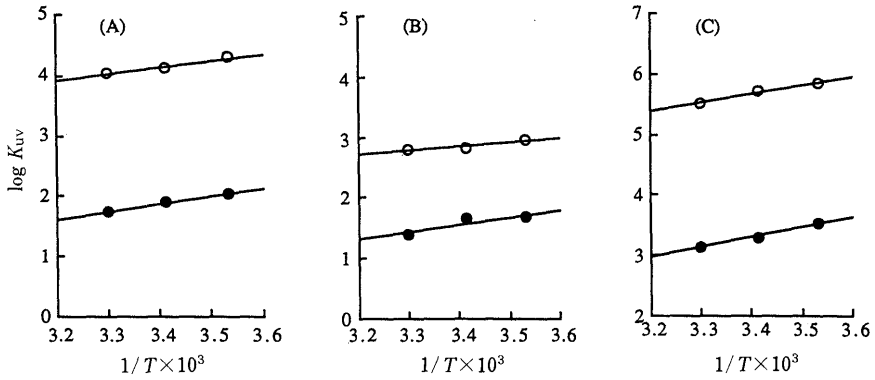

Fig. 4. Typical van't Hoff Plots for Stability Constants of Barbital/α-CD System (A), Phenobarbital/α-CD System (B) and Pentobarbital/α-CD System (C)

○, K_1; ●, K_2.

point depression and the spectroscopic methods measure the same quantity, and the reproducibility and accuracy of the former method are no less than those of the latter method. For example, the relationship between the logarithm of stability constants by the freezing point depression method and that by the spectroscopic method both gave a straight line with a correlation coefficient of 0.99.

Discussion

We developed a simple method to determine the apparent stability constant of 1 : 1 and 1 : 2 complexes, based on the freezing point depression decrease for the barbiturate and α-CD mixture as compared to the sum of those for the individual solutions.

In this study, barbiturate/α-CD complexes were used as a model compound, since a barbiturate is known to form 1 : 1 and 1 : 2 complexes with α-CD. Koizumi *et al.* reported the stoichiometric mole ratios of barbiturate/α-CD complexes. They showed that 1 : 2 complexes were formed between barbiturates and α-CD, while β-CD formed 1 : 1 complexes with barbiturates, according to the solubility analysis of solutions.[8)]

Many methods have been developed to analyze systems containing both 1 : 1 and 1 : 2 complexes, such as solubility, kinetic, spectroscopic, and competitive indicator methods. However, these methods have several advantages, they also have some distinct disadvantages. The procedure described here has several advantages in comparison to the other available methods. It not only applies to 1 : 2 complex systems, but it also indicates the probable stoichiometric ratio. Furthermore, it can directly measure the complex formation without a third compound, as is used in the competitive method. On the other hand, it has some disadvantages: measurement of the stability constant is restricted to being taken only near the freezing point; also, the substrate must dissolve in water to a certain degree (> 10 mM). Despite these limitations, the freezing point depression method has one notable feature: it can rapidly determine the apparent stability constant with a very simple procedure.

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