A Study of Cyclodextrin Complex Formation by a Freezing Point Depression Method[1,2]

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The interaction and the self-association of a variety of compounds in aqueous solution were investigated by the freezing point depression method using a commercially available osmometer. This method, described in our earlier publication, was used to determine the stability constant and self-association constant, which were based on the colligative properties of solution and calculated from the decrease of osmotic pressure obtained in terms of the freezing point depression. Stability constants were measured for complexes of α-, β-, and γ-cyclodextrin with non-aromatic carbonic acids (acetic acid, formic acid, oxalic acid, succinic acid, malic acid, tartaric acid, maleic acid, citric acid), anions (I^-, NO_3^-, ClO_4^-, CNO^-, SCN^-, $Cr_2O_7^{2-}$) and amino acids (leucine, isoleucine, methionine, phenylalanine, tryptophan), and the self-association constant for caffeine was measured. These values gave good reproducibility and rapid estimation with a simple procedure. It was concluded that the freezing point depression method is useful for estimation of the stability constant and the self-association constant for drugs in aqueous solution.

Keywords freezing point depression; stability constant; self-association constant; osmotic property; osmotic pressure; cyclodextrin

In a previous communication,[2] the freezing point depression method was reported to be a very useful method to determine the stability constant for complex formation in aqueous solution. Based on colligative properties, stability constants were calculated from the change of osmotic pressure obtained in terms of freezing point depression using a commercially available osmometer. The data indicated that the stability constants of cyclodextrin (CD) complex with alcohols could be measured with good accuracy. However, no complex forming systems other than the alcohol/CD complex were studied.

Complex formation between CD and non-aromatic carbonic acid, anion, or amino acid can not be detected directly by the conventional spectroscopic technique, because there is no change in the spectroscopic properties. Instead of spectroscopic technique, some authors have conducted the measurement with potentiometry[3,4] or a spectral competitive inhibitor technique.[5] However, these techniques involve operational and theoretical complications.

In the present study, we examined the applicability of the freezing point depression method to some compounds/CD systems and a self-associate system. Osmotic values suggested that carbonic acids (short-chain and non-aromatic carbonic acids), anions (inorganic salts), and amino acids could interact with CD and that caffeine could self-associate in aqueous solution. Furthermore, the stability constants and the self-association constants could be determined by the freezing point depression method. The aim of the present study was to extend the utility of the freezing point depression method.

Experimental

Materials α-, β-, and γ-CD were obtained from Nihon Shokuhin Kako Co., Ltd. Non-aromatic carbonic acids and inorganic salts were from Iwai Kagaku Co., Ltd., and other chemicals were from Tokyo Kasei Kogyou Co., Ltd. All compounds used were of reagent grade. Distilled de-ionized water was used.

Apparatus Osmotic measurements were made using the Osmette Model 2007 (Precision Systems., Inc.), which was calibrated with standard solutions (100, 500 mOsm/kg) of dextrose supplied by the company. The instrument was built according to the principles and practices previously described.[6]

Measurement of Osmotic Concentration All solutions were prepared with distilled water. Osmolality was measured with 2 ml of sample solution and was replicated three times for each solution. The reproducibility of the measurement was reported previously to be within 1% for 50 mM solution.[2]

Theoretical

Determination of Stability Constant The osmotic concentration ($\bar{M}$) is defined as $v \times \phi \times M$, where v is the number of species in solution per molecule of solute, ϕ is the molal osmotic coefficient, and M is the molality of the solute. In this study, calculation involves two assumptions. The first assumption is that $\phi = 1$, and the second is that molality is equal to molarity. These assumptions are applicable to a dilute solution (less than 0.1 molar).[7] Let us assume that solute A and solute B form a 1 : 1 complex AB, whose stability constant K is given by Eq. 1.

$$\begin{aligned} &A + B \rightleftharpoons AB \\ &K = [AB]/[A] \times [B] \\ &\quad = \Delta/[A_o - \Delta] \times [B_o - \Delta] \end{aligned} \tag{1}$$

where A_o and B_o are the total concentrations of solute A and solute B, respectively, [AB] represents the concentration of the complex, and Δ is the difference between stoichiometric molality M and osmotic molality $\bar{M}$; $\Delta = M - \bar{M}$. Here, we call the determined apparent K from freezing point depression K_{osm}.

Determination of Self-Association Constant The self-association process is assumed to occur *via* the formation of a single association species, and according to stepwise equilibrium.

$$\begin{aligned} &A_1 + A_{i-1} = A_i \\ &K_i = [A_i]/[A_1] \times [A_{i-1}] \end{aligned} \tag{2}$$

where $i = 2$ to n, A_1, A_2, $\cdots$, A_n denote the monomer, dimer, $\cdots$, n-mer, respectively, $[A_1]$, $[A_2]$, $\cdots$, $[A_n]$ are the

equilibrium molalities of the respective species, and $K_2, K_3, \cdots, K_n$ is the corresponding partial association constant. In a dilute solution, the stoichiometric molality M and the osmotic molality $\bar{M}$ is expressed as follows;

$$M = A_1 + 2A_2 + 3A_3 + \cdots + nA_n \quad (3)$$

$$\bar{M} = A_1 + A_2 + A_3 + \cdots + A_n \quad (4)$$

The following assumption will be made; the association constants are the same for each successive step; $K_2 = K_3 = \cdots = K_n = K_A$, therefore,

$$A_n = K_A^{n-1} A_1^n \ (n\text{-mer}) \quad (5)$$

$$M = A_1 + 2K_A A_1^2 + 3K_A^2 A_1^3 + \cdots + nK_A^{n-1} A_1^n \quad (6)$$

$$\bar{M} = A_1 + K_A A_1^2 + K_A^2 A_1^3 + \cdots + K_A^{n-1} A_1^n \quad (7)$$

If the solutes associate without any restriction and provided that $K_A A_1 < 1$, Eqs. 6 and 7 can be approximated as follows;

$$M = A_1 (1 - K_A A_1)^{-2} \quad (8)$$

$$\bar{M} = A_1 (1 - K_A A_1)^{-1} \quad (9)$$

Combination of Eq. 8 with Eq. 9, yields the relationship;

$$M/\bar{M} = 1/\phi = K_A \bar{M} + 1 \quad (10)$$

or

$$\bar{M} = (-1 + \sqrt{1 + 4K_A M})/2K_A \quad (11)$$

where ϕ is osmotic coefficient; $\phi = \bar{M}/M$. Eq. 10 therefore provides a graphical approach to determine the self-associate constant K_A by plotting $1/\phi$ against $\bar{M}$, or according to Eq. 11, the estimate of K_A should be possible by a computed technique. The derived Eq. 10 was the same as the transformed equation from the one derived from the Gibbs–Duhem equation by Ts'o and Chan.[8] By using determined K_A values, the weight percentages of monomer were calculated from Eq. 8 and dimer, trimer, $\cdots$, and n-mer could be obtained by Eq. 5.

Results and Discussion

Measurement of Stability Constant The individual osmolalities of α-, β-, and γ-CD, as well as carbonic acid, inorganic salt, and amino acid, and also CD/carbonic acid, CD/inorganic salt, and CD/amino acid in combination, were measured. The final concentration of each solution was adjusted to 50 mM, except β-CD and inorganic salt, whose solutions were adjusted to 10 and 25 mM, respectively.

Substituting experimental values into Eq. 1, the stability constants K_{osm} for 1 : 1 complex formation were determined. Table I summarizes the obtained K_{osm} values for complex formation of various carbonic acids with CD by means of the freezing point depression method. As is seen from the Table, the interaction of α-CD with carbonic acid seems to be an order of magnitude above those obtained for the β- and γ-CD interactions, except for citric acid/β-CD complexes. In particular, γ-CD had little or no interaction with carbonic acids. The large cavity of γ-CD does not seem to be suitable for complexing those carbonic acids. In fact, the extent of interaction (K_{osm}) had a tendency to decrease with increasing CD ring size, except for citric acid/CD complexes.

Interaction of inorganic salt with several CDs was revealed through the freezing point depression method. From the measured interactions between 28 salt species and α-CD, it became clear that: (1) cation species, Li^+, Na^+, K^+, Ag^+, Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, and NH_4^+ did not interact with α-CD; (2) 6 anion species, I^-, NO_3^-, ClO_4^-, CNO^-, SCN^-, and $Cr_2O_7^{2-}$, interacted with α-CD, the K_{osm} values for which are shown in Table II; (3) other anions, *i.e.*, Cl^-, Br^-, SO_4^{2-}, and CO_3^{2-}, did not exhibit interaction.

For the anions that interact with α-CD, the interactions with β- and γ-CD were investigated. In the same manner as α-CD, β- and γ-CD bound those anions, with the exception of the γ-CD/NO_3^- system. The order of magnitude of K_{osm} did not seem to be affected by the ring size of CD regardless of anionic species, although ring size does play

Table I. Stability Constants of CD Complexes with Various Carbonic Acids

Carbonic acid	α-CD (K_{osm}; M^{-1})	β-CD (K_{osm}; M^{-1})	γ-CD (K_{osm}; M^{-1})
Acetic acid	14.5± 1.9	No	No
Formic acid	4.5± 0.9	No	No
Oxalic acid	28.2± 3.3	No	No
Succinic acid	174.3±16.2	19.6± 4.5	2.6±0.6
Malic acid	3.9± 1.7	5.2± 0.0	1.7±0.3
Tartaric acid	1.5± 0.3	7.8± 2.3	1.9±0.0
Maleic acid	12.3± 1.5	[a]	2.1±0.3
Citric acid	13.1± 4.1	50.5±39.2	2.5±1.1

a) Could not be determined with accuracy due to the precipitation formed. No: complex was not formed. Each value represents the mean±S.D. of three determinations.

Table II. Stability Constants of CD Complexes with Various Anions

Anion	α-CD (K_{osm}; M^{-1})	β-CD (K_{osm}; M^{-1})	γ-CD (K_{osm}; M^{-1})
I^-	42.1±12.1	4.6±1.4	7.2±1.7
NO_3^-	8.1± 1.7	56.1±0.0	No
ClO_4^-	83.9±11.1	8.1±1.7	5.4±1.4
CNO^-	4.7± 2.6	1.7±0.0	48.3±6.7
SCN^-	62.6±15.3	5.4±1.4	6.2±0.0
$Cr_2O_7^{2-}$	32.7± 4.5	10.2±2.0	18.3±2.9

Each value represents the mean±S.D. of three determinations. No: complex was not formed.

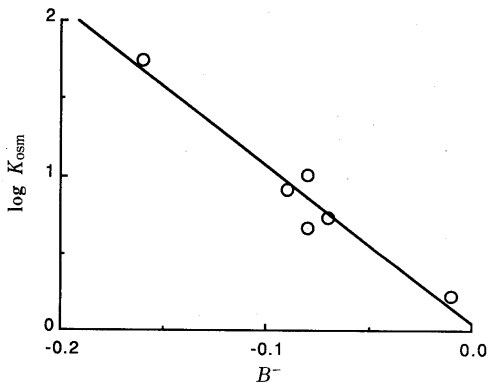

Fig. 1. Relationship between Logarithm of the Stability Constants of Anion/β-CD Complexes Determined by Freezing Point Depression Method and the B^- Values

The B^- values were taken from ref. 5.

TABLE III. Stability Constants of CD Complexes with Various Amino Acids

Amino acid	α-CD (K_{osm}; M^{-1})	β-CD (K_{osm}; M^{-1})	γ-CD (K_{osm}; M^{-1})
L-Leucine	8.7±0.5	No	No
L-Isoleucine	8.4±0.5	No	No
L-Methionine	17.2±0.3	1.5± 1.3	No
L-Phenylalanine	16.3±2.0	21.7±10.1	1.5±0.3
L-Tryptophan	27.8±0.6	>225	14.9±1.1

Each value represents the mean±S.D. of three determinations. No: complex was not formed.

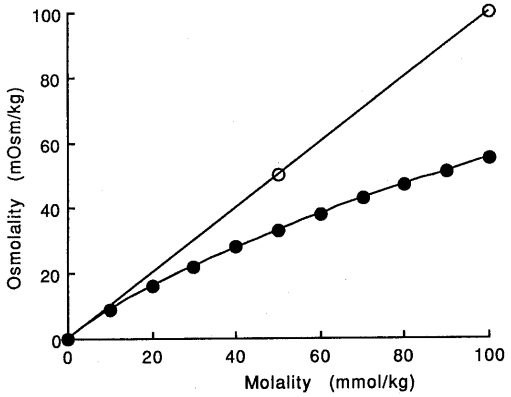

Fig. 2. Plot of Experimental Osmolality *Versus* Stoichiometric Molality of Caffeine (●) and Dextrose (○) in Aqueous Solution

a significant role in hydrophobic binding.[9] The anion molecules are so small that there is no steric hindrance, and therefore the stability constants are not affected by the CD species. Rohrbach *et al.*[5] reported that the formation of salt/CD complex is related to the proton nuclear magnetic resonance relaxation rate, B^- value, or the "structure breaking" properties of anion. In the same manner as above, our data (Fig. 1) were also found to exist in a correlation between the log of the K_{osm} for the various salt/β-CD complexes and the B^- value. This indicated that a quantitative correlation with some water sensitive parameter might exist.[5]

Table III shows the K_{osm} of amino acid/CD interaction. The interactions in 16 amino acid/CD systems were measured by the freezing point depression method. The examined amino acids are divided into the following groups: (1) amino acids with non-polar R (side chain) groups, (2) amino acids with uncharged polar R groups, (3) amino acids with negatively charged (acidic) R groups, and (4) amino acids with positively charged (basic) R groups.

Only hydrophobic amino acids with non-polar R groups in all of the above amino acids interact with CDs, except for alanine, valine, and proline. The stability constant K_{osm} decreased in the order Trp > Phe ≧ Met ≧ Leu = Ile. These results suggest that the interacting force of CD is influenced by the hydrophobic and the steric factor of R group of amino acid.

Measurement of Self-Association Constant The osmotic molalities of caffeine over the concentration range of 0.01 to 0.1 mol/kg in aqueous solution were measured at the freezing point depression with an osmometer that was calibrated with dextrose. The relationship between experimental molality as well as the calculated osmotic molality (solid line) and stoichiometric molality, is presented in Fig. 1. The match of experimental osmotic molality with theoretical curve was recognized.

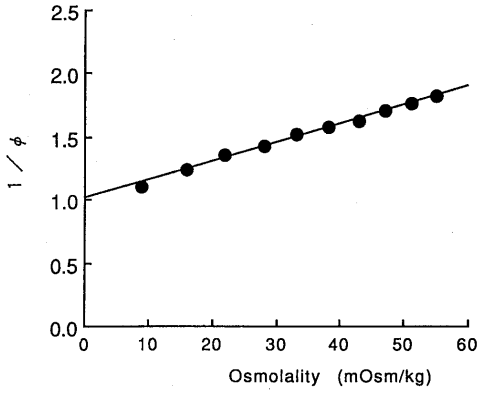

Fig. 3. Relationship between Calculated Values for $1/\phi$ and Experimental Osmolality of Caffeine in Aqueous Solution

TABLE IV. Weight Percentage of Monomer, Dimer, Trimer, Tetramer, and Pentamer at Each Concentration of Caffeine

Caffeine conc. (mM)	Weight percentage (%)				
	Monomer	Dimer	Trimer	Tetramer	Pentamer
10	78	18	3	1	0
50	44	30	14	7	3
100	30	27	18	11	8

Weight percentage (%) was calculated according to Eqs. 5 and 8.

Figure 2 shows the plot of $1/\phi$ against $\bar{M}$ according to Eq. 10, where osmotic activity coefficients ϕ were calculated from the experimental osmotic molalities. Such a straight-line plot means each step in the association process is equally favorable and without any restriction on the polymer size. The association constant K_A can then be determined either from the slope of the linear plot according to Eq. 10 or by the computed method (Eq. 11) using nonlinear regression. From fitted data, the association constant of caffeine obtained was $15\,M^{-1}$. By using the measured K_A values, the contents by weight percentage of each associated species were estimated and are summarized in Table IV. It is seen that, in the case of caffeine at 100 mM, the weight percentage of monomer is only 30%. Most of caffeine molecules are found in associated forms.

Advantages and Disadvantages of the Osmotic Method In this report, we showed the applicability of the freezing point depression method to some drug/CD systems and a self-associate system. As mentioned above, this osmotic method has the following advantages and disadvantages. Advantages are: (1) the measurement can be conducted with a simple procedure within a short time and with good reproducibility; (2) it can be applied to a combination for which quantitative measurements on complex formation by conventional methods, such as absorbance change, are impossible. On the other hand, disadvantages are: (1) substrate must dissolve in water to a certain degree (>10 mM); (2) obtained K_{osm} values should be relatively small; (3) it can be determined only at around at 0 °C, *i.e.* the freezing point. Despite these limitations, the osmotic

method is concluded to be useful. It can give not only a rapid estimation of whether or not an interaction exists, but also determination of the stability constant and the self-association constant.

References and Notes

1) A part of this work was presented at the 109th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1988.
2) This paper constitutes Part II of the studies entitled "Application of Freezing Point Depression to Drug Interaction Studies. I." Part I: M. Suzuki, S. Ueda and A. Kusai, *Chem. Pharm. Bull.*, **36**, 720 (1988).
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