Ultraviolet Spectroscopic Estimation of Microenvironments and Bitter Tastes of Oxyphenonium Bromide in Cyclodextrin Solutions

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Abstract □ The UV absorbance and bitter taste of oxyphenonium bromide (OB), an antiacetylcholine drug, in cyclodextrin (CD) solutions are measured, and the local environment of the binding site and the reduction of the bitter taste intensity are quantitatively estimated from the UV data. The UV spectrum of OB is changed with the addition of α-, β-, and γ-CD, because the phenyl group of OB is included into the CD cavity. The maximum wavelength, λ_{max}, senses environmental changes of OB best among several spectral characteristics. From comparison of λ_{max} between a CD solution and the reference ethanol–water and dioxane–water systems, the dielectric constant of the binding site is evaluated. This value leads us to estimate the microenvironment and structure of the binding site. The suppression of the bitter taste of 4 mM OB by CDs is in the increasing order α-CD < γ-CD < β-CD. The extent of this suppression can be quantitatively predicted from the UV absorbance by assuming that the free OB molecule alone exhibits the bitter taste, regardless of the kind and concentration of CD. Some implications and limitations of the present approach are discussed.

Introduction

Cyclodextrins (CDs) have homogeneous toroidal structures of different sizes. One side of the torus contains primary hydroxyl groups, whereas the secondary groups are located on the other side. The toroidal structure has a hydrophilic surface resulting from the 2-, 4-, and 6-position hydroxyls, making them water-soluble. The cavity is composed of the glucoside oxygens and methylene hydrogens, giving it a hydrophobic character.[1,2] CDs can give beneficial modifications of guest molecules not otherwise achievable: solubility enhancement, stabilization of labile guests, control of volatility and sublimation, and physical isolation of incompatible compounds. Because they are practically nontoxic, they are added into pharmaceuticals and foods for stabilization of labile compounds and long-term protection of color, odor, and flavor.[2,3] Furthermore, cyclodextrins can mask bitter tastes of drugs,[2,3] e.g., propantheline bromide[4] and oxyphenonium bromide.[5]

The effect of solvents on the ultraviolet (UV) spectra of aromatic molecules, particularly benzene, has been studied extensively. UV spectra are often sensitive to the nature of the local environment in solution.[6] Medium sensitive spectral characteristics can in turn be useful for studying the microenvironments of molecules and, indeed, a great variety of spectroscopic methods in solutions of small molecules and macromolecules as also in micelle and membrane research.[7] Many such studies have the objective of estimating the polarity of the microenvironment by comparison with a number of reference solvents. Many times relatively small changes with CD inclusion can be observed on UV spectra of guest.[2,3] At least an approximate estimation of the polarity of the internal CD cavity enables us to understand the inclusion of the various guests and to predict the type of the potential guests. Various and different estimates have been published. One estimate is based on the fact that the spectral shift shown by the *N,N*-diphenylamine (DPA) fluorescence probe depends on the polarity of ethanol–water mixtures. The fluorescence spectra of DPA show that the polarity of β-CD seems to be identical with that of a 40% ethanol/water mixture. Of course, this value cannot be considered to be an absolute one, but probably only as a lower level. The DPA molecule in solution is completely surrounded by solvent molecules, while the extent of penetration of the DPA guest into the CD cavity is probably only a partial inclusion.[3] From measurements of fluorescence quantum yields, Kondo et al. estimated the dielectric constant of 6-*p*-toluidinylnaphthalene-2-sulfonate in the CD cavity.[8]

Sensory tests generally depend on individuals. Some instrumental methods, therefore, are desired for such tests. In this work we develop a UV method for the quantitative estimation of the bitter taste intensity of oxyphenonium bromide (OB, Figure 1), an antiacetylcholine drug, in aqueous solutions of α-, β-, and γ-CDs. The UV absorbance can be used for the determination of the binding constants of OB with the CDs. Those constants enable us to estimate the concentration of the free OB molecule in the CD solutions. Bitter compounds are generally hydrophobic,[9] but their CD complexes are rather hydrophilic, because of the hydrophilicity of CDs.[4] Thus we can expect that the bitter taste of OB in a CD solution is estimated from the free OB concentration alone.[4,5] The other research interest is that OB has two possible hydrophobic groups for CD inclusion, phenyl and cyclohexyl groups. The incorporation of the phenyl group into the CD cavity should result in UV spectral changes, because of the change of its microenvironment. Thus, we can expect to determine which group of OB is entrapped into the CD cavity.

Experimental Section

Materials—A sample of OB was purchased from Sigma Chemical Co. Because this sample was analyzed to be pure by reversed-phase liquid chromatography, it was used without purification. Sodium bromide of analytical grade and α-, β-, and γ-CDs from Nacalai Tesque Co. (Kyoto) were used as received. The ion-exchanged water was used after double distillation.

UV Measurements—All UV spectra were recorded with a Hitachi U-3000 spectrophotometer at 36.5 ± 0.1 °C. The absorbance was determined at an interval of 0.5 nm between 220 and 300 nm and at an interval of 0.1 nm between 240 and 280 nm. The first and second derivative spectra of OB were recorded at an interval of 0.1 nm between 240 and 280 nm. All sample and reference cuvettes contained 154 mM NaBr, unless specified. The sample cuvette contained 4 mM OB and CD, whereas the reference cuvette contained CD of the same concentration as the sample. The difference spectra were obtained by subtracting the spectra of a 4 mM OB solution from those of sample solutions. The maximum wavelength, λ_{max}, was determined from the first deriva-
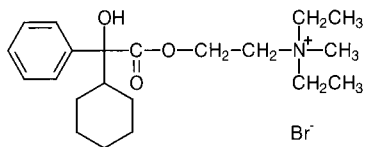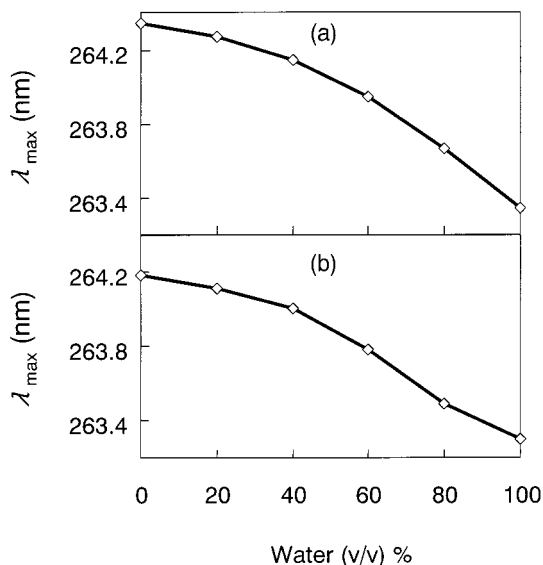
Figure 1—Chemical structure of OB.

Figure 2—UV maximum wavelength of OB vs volume percent of water in binary mixtures of (a) ethanol and water and (b) dioxane and water. The smooth lines are hand-drawn through the observed data at 36.5 °C.

tive spectrum. The CD concentrations in the sample were changed up to 50 mM, 10 mM, and 50 mM for α-CD, β-CD, and γ-CD, respectively. For each CD system the difference spectra were obtained at about seven concentrations, and those absorbance data were used for the determination of the binding constant of OB and CD.

The effects of ethanol and dioxane on the spectrum of 4 mM OB were investigated at several compositions without NaBr.

Test of Bitter Taste—Five volunteers were involved in the sensory test. These panelists tasted aqueous 154 mM NaBr solutions containing OB alone and a mixture of 4 mM OB and CD. Each volunteer categorized the bitter taste intensity of a solution as one of the following scores: 0 = no bitter taste; 1 = very slightly bitter taste; 2 = slightly bitter taste; 3 = appreciably bitter taste; 4 = very bitter taste; 5 = extremely bitter taste. The bitterness score for the solution was averaged over the five individuals, so that the averaged score of the solution was a continuous variable smaller than 5.

Data Treatments—Absorbance data were transferred to tables in Microsoft Excel 97 to be subject to further analysis. The nonlinear least-squares method available in Microsoft Excel 97 was used.

Results and Discussion

UV Spectra of Oxyphenonium Bromide—The aromatic chromophore absorbs the UV light. The water spectrum of benzene, for example, exhibits a bathochromic shift, a reduction in the peak intensity, and a broadening of the bands.[6] Similar spectral changes were observed for OB in water, dioxane, ethanol, and their mixtures. The maximum wavelength (λ_{max}), minimum wavelength (λ_{min}), and absorbance ratio (A_{max}/A_{min}) at λ_{max} and λ_{min} in original spectra and corresponding spectral parameters in the first and second derivative spectra depend on the solvents. The peak around 263 nm is the most sensitive to the solvent among these spectral parameters. As Figure 2 shows, this band shifts toward the longer wavelength side with the addition of ethanol and dioxane.

Table 1—Absorption Maximum Wavelengths, Dielectric Constants, and Binding Constants of OB in CD Solutions at 309.7 K

CD	λ_{max} (nm)	D_{ETOH}	$D_{ETOHcor}$[a]	D_{DIO}	D_{DIOcor}[a]	K_1 (M^{-1}) UV	K_1 (M^{-1}) emf[b]
none	263.30	74.5	74.5	74.5	74.5	–	–
50 mM α-CD	263.48	66.5	64.7	66.5	64.7	94	58
10 mM β-CD	264.31	<22.7	<22.7	4.5	2.9	7350	8500
50 mM γ-CD	263.98	45.0	40.5	36.5	30.7	140	96

[a] Dielectric constant at full binding. [b] Electromotive force measurements.[5]

As Table 1 shows, the CDs also cause red shifts. This result indicates that OB is bound to these CDs. Comparison between these λ_{max} values and the results of Figure 2 enable us to estimate the local microenvironments of OB in the CDs. The dielectric constants of the water−ethanol[10] and water−dioxane[11] systems at 37 °C are available in the literature. The estimated dielectric constants are shown at columns labeled D_{ETOH} and D_{DIO} in Table 1. The dielectric constant in a 10 mM β-CD solution is smaller than that of ethanol (22.7).[10] These dielectric constant values do not accurately correspond to the true microenvironments of the binding sites, because all of the OB molecules are not incorporated into the CDs under the present conditions. To correct this partial binding, we need the binding constants of OB with the CDs.

Binding Constants of Oxyphenonium Bromide for Cyclodextrins—On the basis of electromotive force data, we have shown that OB (P) does not self-associate below 10 mM and that it forms the 1:1 complexes (PD) alone with α-CD, β-CD, and γ-CD (D).[5] The equilibrium constant of this complexation is defined as

$$K_1 = [PD]/[P][D] \qquad (1)$$

The total concentrations of OB and CD are written as

$$C_P = [P] + [PD] \qquad (2)$$

$$C_D = [D] + [PD] \qquad (3)$$

From eqs 1−3, we can obtain the concentration of free OB:

$$[P] = \{K_1 C_P - K_1 C_D - 1 + [(K_1 C_P - K_1 C_D - 1)^2 + 4K_1 C_P]^{1/2}\}/2K_1 \quad (4)$$

Figure 3 shows typical difference spectra of OB in solutions of α-CD, β-CD, and γ-CD containing 154 mM NaBr. At a given wavelength the absorbance difference, ΔA, can be expressed as

$$\Delta A = l(\epsilon_{PD} - \epsilon_P)[PD] \qquad (5)$$

$$= l(\epsilon_{PD} - \epsilon_P)\{K_1 C_P + K_1 C_D + 1 - [(K_1 C_P - K_1 C_D - 1)^2 + 4K_1 C_P]^{1/2}\}/2K_1 \quad (6)$$

where ϵ_P and ϵ_{PD} denote the molar absorptivities of OB and the equimolar complex of OB and CD, respectively, and l stands for the optical path length of the cuvette. The ϵ_P value was determined from the spectrum of a 4 mM OB solution.

From eq 6 we can calculate a theoretical absorbance difference value for a given set of C_P and C_D. The values of ϵ_{PD} and K_1 were regarded as adjustable parameters to be best fitted to the observed absorbance difference by nonlinear least-squares method. The best fit binding constants are shown in Table 1. These values are close to those
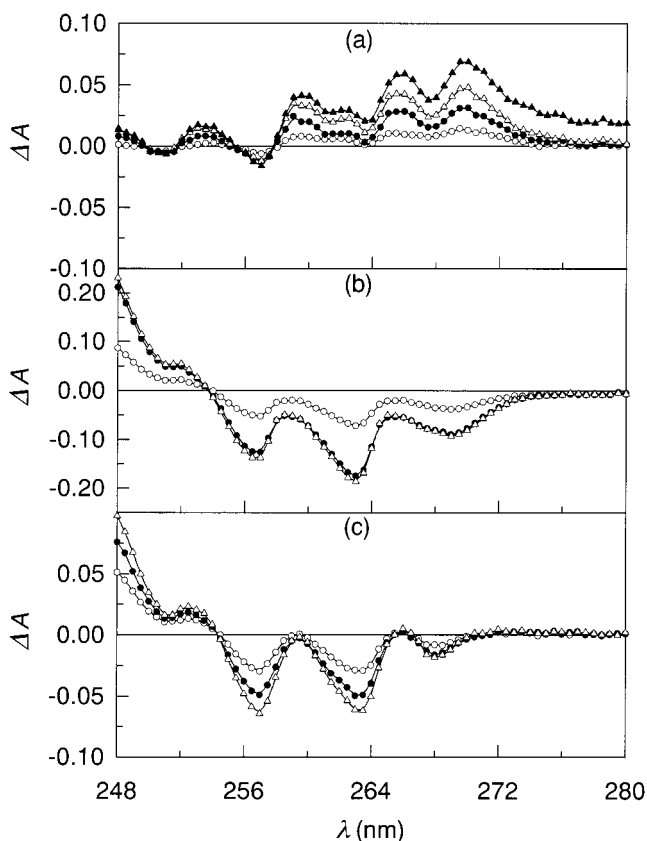
Figure 3—Typical UV difference spectra of 4 mM OB in 154 mM NaBr solutions of (a) α-CD, (b) β-CD, and (c) γ-CD. The CD concentrations (mM) were at 5 (○), 10 (●), 20 (△), and 30 (▲) for α-CD, 1.5 (○), 4.5 (●), and 9.0 (△) for β-CD, and 5 (○), 10 (●), and 15 (△) for γ-CD.

Table 2—Molar Absorptivities (cm⁻¹ mM⁻¹ at Two Wavelengths) of OB and the Equimolar Complex, the Number of Data, and the SS Value for the OB–CD Systems at 309.7 K

CD	ϵ_P	ϵ_{PD}	λ_1 (nm)	ϵ_P	ϵ_{PD}	λ_2 (nm)	n	SS × 10⁴
α-CD	0.1596	0.1752	265.5	0.0821	0.1001	269.5	12	7.79
β-CD	0.2277	0.1945	257.0	0.1886	0.1418	263.0	13	2.49
γ-CD	0.2277	0.2030	257.0	0.1917	0.1669	263.5	13	2.15

determined by electromotive force measurements.[5] For α-CD the observed absorbance differences at 265.5 and 269.5 nm were used simultaneously, because these values exhibited the greatest changes. By the same reasons the wavelengths at 257.0 and 263.0 and at 257.0 and 263.5 nm were employed for β-CD and γ-CD, respectively. The extent of fitting was measured by the SS value defined as

$$SS = \sum^{n} (\Delta A_{obsd} - \Delta A_{calcd})^2 \qquad (7)$$

The number, n, of data and the SS value were shown in Table 2, together with the molar absorptivities for the equimolar complex of OB and CD.

Now we can estimate the dielectric constants at the binding sites of OB in CD by using the binding constants obtained by the UV method. The binding percent of OB is 81.5% for 50 mM α-CD, 97.8% for 10 mM β-CD, and 86.8% for 50 mM γ-CD. We assumed that the estimated dielectric constant is the sum of the free OB and bound OB. Thus, we can estimate the dielectric constants, D_{cor}, at full binding for the ethanol and dioxane systems. As Table 1 shows, these dielectric constants for CD are rather close to one another for the two solvent systems. This fact
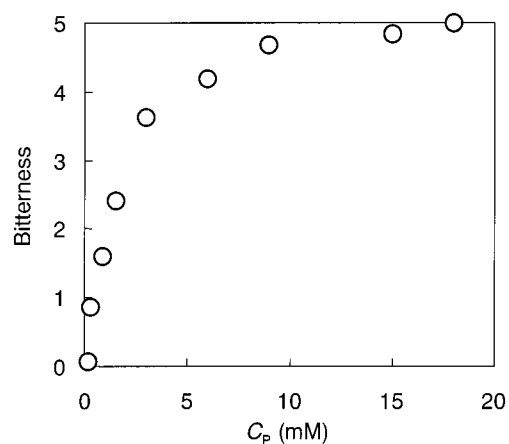

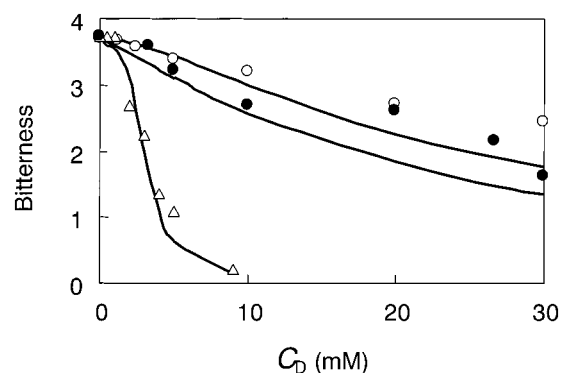**Figure 4**—Scores of bitter tastes of aqueous OB solutions plotted against the OB concentrations.

Figure 5—Effects of CDs on the bitter taste intensity of a 4 mM OB solution: α-CD (○), β-CD (△), and γ-CD (●). The solid lines are calculated from eqs 4 and 8 by using the equilibrium binding constants shown in Table 1.

demonstrates the validity of the present estimation. The dielectric constant for α-CD suggests that the phenyl group of OB is outside of the α-CD cavity or is shallowly penetrated into the cavity. The phenyl group of OB will be deeply incorporated into the β-CD cavity, because an estimated dielectric constant of $D_{DIOcor} = 2.9$ is very close to 2.1 of dioxane.[11] Because this group is snugly incorporated in the γ-CD cavity, the cavity has room enough to contain some remaining water molecules. Therefore, OB senses the γ-CD cavity to be a mixture of dioxane and water with $D_{cor} = 30.7$.

Thus, we can estimate the microenvironments of OB in the CDs from the UV spectra.

Effects of Cyclodextrins on the Bitter Taste Intensity of OB—The intensity of bitter taste of an OB solution is shown as a function of OB concentration in Figure 4. As Figure 5 shows, the bitter taste intensity of a 4 mM OB solution is remarkably reduced by the addition of CDs. This reduction by the kind of CD is in the increasing order of α-CD < γ-CD < β-CD, consistent with the magnitude of the binding constant.

Bitter compounds have a variety of chemical structures including alkaloids and terpenes. These compounds, however, are generally hydrophobic and easily absorbed to the receptor of bitter sites.[9] OB tastes bitter, but CD is not a bitter compound, because of its hydrophilicity. We have shown that the complexes of CD with surfactants[12] and propantheline bromide[4] are surface-inactive. Similarly, the complex of OB and CD is rather hydrophilic and would not taste bitter.[4,5] Thus we can assume that the free OB molecule alone exhibits the bitter taste, regardless of its CD complex and CD. On the basis of this assumption, the bitter taste intensity of an aqueous solution of OB and CD

will be determined by the concentration ([P]) of uncomplexed OB in the solution, irrespective of the kind and concentration of CD:

$$\text{bitter taste intensity} = g\{[P]\} \qquad (8)$$

Actually we did not attempt to fit any empirical equation to the observed bitter scores shown in Figure 4, but drew a smooth line through these data, albeit not shown therein. This line was used for reading a bitter score at a free concentration of OB and is expected to hold true for all solutions of OB and CD.

To verify this assumption, we calculated a theoretical bitter taste intensity by using eqs 4 and 8 for a solution containing 4 mM OB and CD. Here the binding constant estimated by the UV method was employed in eq 4. The solid curves in Figure 5 show such theoretical values. These values are close to the observed ones. Thus, eq 8 holds true for all solutions containing OB and CDs. This important result enables us to predict the intensity of bitter taste of an OB and CD solution from the observed UV absorbance of their mixed solution.

Implications and Limitations of the Present Work— The present UV approach for estimating the microenvironment of the binding site will apply for CD inclusion of other chromophores. OB exclusively forms the 1:1 complexes with α-, β-, and γ-CD.[5] Other guests often form ternary and quaternary complexes.[12] For such complicated inclusion systems, the present approach will not provide clear information on their microenvironments, though other spectroscopic methods, such as NMR and circular dichroism, can give more detailed information. Such spectroscopic data will generally provide a basis of the present UV method for estimating the binding site.

The UV method for predicting the bitter taste of OB reduced by CDs will apply to other bitter aromatic compounds. This enables us to determine the kind and concentration of CD to suppress the bitter taste of drug from a few and reproducible experiments. The UV method, however, does not apply for the self-associating or multiple complexing systems, because the UV absorbance senses all of the monomer and polymers of guest and its CD complexes. For the same purpose we have proposed the surface tension[4] and electromotive force[5] methods. Many drugs self-associate in aqueous media by hydrophobic interactions,[13] though OB does not self-associate.[5] Guest generally forms multiple complexes with CD.[12,14] The surface tension and electromotive force methods can apply to such self-associating or multiple complexing systems,[4,5] because these methods sense the free drug molecule alone. The UV method, however, has the advantage of convenience over these methods. This method would apply to bitter blockers with characteristics similar to CD. A kind of lipoprotein can specifically inhibit bitter taste by masking the receptor site for bitter substances.[15] The present method will be inapplicable to such masking systems.

References and Notes

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