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Characterization of the complexation of diflunisal with hydroxypropyl-β-cyclodextrin

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Abstract

The equilibrium dialysis method was applied to the determination of drug–cyclodextrin stability constants using diflunisal and 2-hydroxypropyl-β-cyclodextrin (HPBCD) as a model system. Analysis of the data showed the existence of a linear Scatchard plot, indicative of the formation of a 1:1 diflunisal:HPBCD complex. The mean complexation constant (K_c) $\pm$ S.D. was 3892 ± 360 M^{-1}. The stoichiometry of the complex was verified using the appropriate mass action law equation. The diflunisal:HPBCD complex was also investigated using titration microcalorimetry. A K_c of 3394 M^{-1} was obtained together with an enthalpy change (ΔH) of -20.76 kJ mol^{-1}. The K_c values obtained here using the equilibrium dialysis and microcalorimetric methods were comparable to one reported previously using a potentiometric method (5564 ± 56 M^{-1}).

Keywords: Stability constants; Complexation; Dialysis; Microcalorimetry; Diflunisal; Cyclodextrin

1. Introduction

Cyclodextrins are oligomers of glucose that possess several desirable physicochemical properties. 2-Hydroxypropyl-β-cyclodextrin (HPBCD) is a water-soluble derivative of β-cyclodextrin which has been widely studied as a complexing agent for many pharmaceuticals. Complexation of drugs with HPBCD has been shown to increase their solubility and stability in aqueous solutions [1–3]. Furthermore, toxicities associated with parenterally formulated, poorly water-soluble drugs can often be reduced by formation of soluble drug:HPBCD complexes [4].

The first step in the characterization of drug:HPBCD complexes involves the determination of the binding (stability) constants of the complexes. This parameter is important in the evaluation of the affinity and the degree of binding of drug molecules to HPBCD. A method frequently used for the determination of stability constants is the phase-solubility technique [5]. Although this is a fairly simple method experimentally, it has several shortcomings. This method involves saturation solubility measurements of the drug at different ligand concentrations. As a result, the method is poorly suited for determining

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the stability constants of expensive drugs because relatively large amounts of drug are required to saturate the solutions used to obtain solubility measurements. In addition, it is very difficult to control the pH of saturated solutions with commonly used buffers, and changes in pH can alter the complexation equilibrium. Furthermore, since a constant amount of drug in excess of the solubility is used, the effect of varying drug concentrations on the stoichiometry of the binding process cannot be studied. Finally, it is not always possible to accurately determine unbound and bound drug, and ligand concentrations by this method.

The equilibrium dialysis method has been employed in several studies investigating the binding of drugs to proteins and other macromolecules [6]. However, this method has not been widely applied to the characterization of drug binding to cyclodextrins. Equilibrium dialysis has several advantages over the phase solubility method. Saturated drug solutions are not required for this method, therefore, only small amounts of drug are needed. It is also quite easy to control the pH and ionic strength of true solutions compared to saturated solutions. This method makes it possible to study the effects on complexation of varying either the drug or ligand concentration. This technique also avoids some of the problems associated with the accurate determination of S_o (the solubility of the drug in the absence of ligand) reported elsewhere [7]. Furthermore, it is possible to measure free drug, bound drug, and ligand concentrations. Since the concentrations of free drug, bound drug and ligand are known, the stability constant can be calculated directly without resorting to data transformation. Bruce et al. [8] studied the permeation rates of benzoic acid using a semipermeable dimethylpolysiloxane (PDMS) membrane. From their permeation data the stability constant of the cyclodextrin:benzoic acid complex was estimated. Although the above method is similar in principle to dynamic dialysis, it was more time-consuming and required expensive instrumentation.

In the present study, the equilibrium dialysis method was applied to the determination of the stability constant of HPBCD:diflunisal complexes. The binding constant of the diflunisal:HPBCD complex was also determined using titration microcalorimetry. Diflunisal was selected as the model drug for this study since the binding parameters for this complex were recently evaluated using the potentiometric method and an ion-selective electrode [9], and thus could be compared to the parameters obtained in this study.

2. Experimental

2.1. Materials

The materials used in this study were as follows: potassium phosphate, monobasic crystals (Lot 3246-01), and potassium phosphate, dibasic crystals (Lot 7088KLYT, J.T. Baker, Phillipsburg, NJ), diflunisal (5[2,4-difluorophenylsalicylic] acid) (Lot 56F0118, Sigma, St. Louis, MO), Encapsin HPB (2-hydroxypropyl-β-cyclodextrin (HPBCD)) (Lot # EN92-3, D.S. = 4.10, MW = 1372.8, American Maize Products, Hammond, IN), sodium hydroxide (Lot SX0590-1, MCB Manufacturing Chemists, Cincinnati, OH), methanol (Lot UN 1230, Burdick & Jackson, Muskegon, MI), acetonitrile (Lot NA1648, Burdick & Jackson), potassium chloride (Lot 3040-01, J.T. Baker), Capran 77C nylon membrane, 0.6-mil (Allied Chemical, Morristown, NJ), acrylic plastic dialysis cells (Model 289, Bel-Art Products, Pequannock, NJ), Filters, 0.45-μm (Lot HAWP 013 00, Millipore Corp., Bedford, MA). All solutions were prepared using deionized and distilled water from a Millipore filtration system (Millipore).

2.2. HPLC analysis

The HPLC system consisted of an Altex (Altex Scientific, Berkeley, CA) Model 110A pump, a Rheodyne (Cotati, CA), Model 7125 injector with a 50-μl loop and a Hitachi/Spectra-Physics (Fremont, CA) Model 100-30 variable-wavelength UV detector set at 262 nm. The analytical column was a Phenomenex (Torrance, CA) C-18 column (10 μm, 300 × 3.9 mm i.d.) fitted with a Whatman (Clifton, NJ) C_{18} (30 μm) guard column (10 × 4.6 mm). Peak recording and height measurements were made with a Kipp & Zonen (Bohemia, NY)

Model BD-40 recorder. The mobile phase was the same as that reported previously [10]. It consisted of 58% (v/v) of 0.01 M phosphate buffer, pH 7.0; 26.3% (v/v) acetonitrile; 15.7% (v/v) methanol. A flow rate of 1.0 ml min^{-1} was utilized. Duplicate 50-μl injections were made for each sample using a Hamilton (Reno, NV) Model 702-SNR 100-μl syringe.

2.3. Titration microcalorimetric studies

Microcalorimetric techniques have been used previously to study the complexation of drugs with HPBCD [11]. Titrations were performed using the Thermometric model 2277 Thermal Activity Monitor (TAM) isothermal microcalorimeter (Thermometric, Järfälla, Sweden). The calorimeter was maintained at 25 ± 0.0001°C. Diflunisal (0.9 ml, 0.897 mM) in phosphate buffer (0.01 M, pH 7.4) was placed in the reaction vessel and stirred constantly at approximately 50 rpm. Hydroxypropyl-β-cyclodextrin (8.945 mM) was introduced into the vessel from a Hamilton Microlab M microsyringe pump equipped with a 250-μl syringe. Twenty-five equal aliquots (10 μl each) of the cyclodextrin solution were injected into the reaction vessel containing the diflunisal. The time interval between each injection was 5 min. After each injection, the heat effect was observed until the exotherm returned to the baseline. To determine the heat flow caused by the dilution of the cyclodextrin solution a similar experiment was performed by placing the vehicle only (i.e. no diflunisal present) in the reaction vessel and injecting aliquots of cyclodextrin solution into it in the same manner as in the sample titration. The heat flow due to dilution (about 120 μJ per injection) was subtracted from the heat flow generated during the actual sample titration. The data were analyzed using Thermometric's DIGITAM, V 3.0 data analysis software system.

2.4. Equilibrium dialysis studies

The Capran nylon membranes were washed for 1 h in deionized, distilled water to remove any contaminants [12]. The dialysis cells were assembled with the membrane acting as a semipermeable barrier between the two compartments. Then, 0.8 ml of phosphate buffer solution (0.01 M, pH 7.4) was added to one cell compartment (the aqueous compartment) and 0.8 ml of solutions consisting of varying volume ratios of diflunisal (0.01 M) in HPBCD (8.974 mM) and HPBCD (8.974 mM) were added to the other cell compartment (ligand compartment). The cells were placed on a water bath shaker (GCA/Precision Scientific, Chicago, IL) and agitated at 100 oscillations min^{-1} for 21 h at room temperature (23–25°C) until equilibrium was achieved. Samples were removed from both cell compartments and analyzed for diflunisal using the above HPLC method.

The equilibration time was determined by measuring diflunisal concentrations at 4, 8, 21 and 48 h. Concentrations of diflunisal at 21 and 48 h were identical, thus a 21-h equilibration time was chosen for the study. The impermeability of the nylon membrane to HPBCD was verified by placing 0.8 ml of buffer solution in one cell compartment and 0.8 ml of 8.974 mM HPBCD solution in the other compartment. After equilibration (21 h), both compartments were assayed for HPBCD using a previously reported method [13]. Possible changes in the volume of the two compartments, due to an osmotic pressure gradient [14], were insignificant during the period of the study. This was determined by measuring the HPBCD concentrations before and after dialysis. Binding of diflunisal to the nylon membrane was determined to be insignificant ($< 0.5\%$).

2.5. Data analysis

In the equilibrium dialysis studies, the concentration of diflunisal measured on the buffer side of the cell compartment represents the free (unbound) concentration, and the difference in diflunisal concentration on the buffer and binder sides of the cell compartments represents the bound diflunisal concentration. The binding of diflunisal to HPBCD and the calculation of the associated binding parameters were analyzed by the methods of Scatchard [15] and Plumbridge et al. [16].

Assuming that only one class of sites exists, and that there are n independent and equivalent bind-

ing sites per molecule of cyclodextrin, each having a complexation constant (K_c), the following equation may be written:

$$r/D_f = K_c(n - r) \quad (1)$$

where r is the average number of moles of diflunisal bound per mole of HPBCD, D_f is the concentration of free (unbound) diflunisal, n is the number of binding sites per molecule of HPBCD, and K_c is the complexation constant for the binding of diflunisal to HPBCD. In many binding studies Eq. (1) has been used to determine n and K_c by regressing r/D_f on r. In accordance with the recommendations of Plumbridge et al. [16] the binding parameters (n, K_c) reported here were obtained by nonlinear regression of D_f (the free or unbound drug concentration) on D_t (the total drug concentration) according to the following equation which is derivable from the law of mass action:

$$D_f = D_t(1 + K_cD_f)/(1 + K_cD_f + nK_cC_{Lt}) \quad (2)$$

where C_{Lt} is equal to total ligand (HPBCD) concentration.

3. Results

Four HPLC calibration curves for diflunisal analysis were obtained. The calibration curves were all linear between 5 and 50 μg ml^{-1} diflunisal ($r \geq 0.9999$). The assay precision was determined by calculating the percent coefficient of variation (%CV) of the slopes obtained on four consecutive days. The %CV of the slopes was 2.8%. Spectral analysis of a 10 μg ml^{-1} concentration of diflunisal at various concentrations of HPBCD showed that there were no complexation-induced spectral shifts [17] which could confound the assay results.

The data obtained from Determination 1 (see Table 1) of the equilibrium dialysis experiments is shown as a Scatchard plot in Fig. 1. The regression of r/D_f vs r produced a highly significant linear correlation ($P < 0.0005$). The observed linearity of the plot is indicative of the presence of one class of binding sites and the adherence of the binding data to Eq. (1). The calculated binding parameters for four separate experiments are summarized in Table 1. The mean ($\pm$ S.D.) values for K_c and n, which were calculated from the regression of D_f on D_t are 3892 $\pm$ 360 M^{-1} and 0.897 $\pm$ 0.042, respectively. The %CV for K_c and n were 9.25 and 4.65, respectively. The values for K_c and n obtained using the more traditional Scatchard analysis, i.e. by regressing r/D_f vs r, are listed in Table 1 for comparison purposes.

The titration microcalorimetry experiments indicated that the interaction of diflunisal with HPBCD was exothermic ($\Delta H = -20.76$ kJ mol^{-1}) with a complexation constant (K_c) of 3394 M^{-1}, and an n of 1.04. The free energy change (ΔG) for the complexation reaction was calculated as:

$$\Delta G = -RT \ln K_c \quad (3)$$

while the entropy change for the binding process was derived from the following expression:

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

From these expressions one can calculate a free-energy change (ΔG) of -20.14 kJ mol^{-1} and an entropy change (ΔS) of -2.06 J deg^{-1} mol^{-1}.

4. Discussion

The stability constants for the diflunisal–HPBCD interaction determined by the equilibrium dialysis, titration microcalorimetric, and potentiometric methods were comparable. As noted by

Table 1
Binding parameters for diflunisal–HPBCD complexation by equilibrium dialysis using two fitting procedures

Determination	K_c (M^{-1})		n	
	r/D_f vs r	D_f vs D_t	r/D_f vs r	D_f vs D_t
1	4593	4030	0.846	0.884
2	4125	4137	0.882	0.885
3	4079	4045	0.854	0.861
4	3962	3357	0.879	0.957
Mean	4190	3892	0.865	0.897
S.D.	277	360	0.018	0.042
%CV	6.62	9.25	2.07	4.65

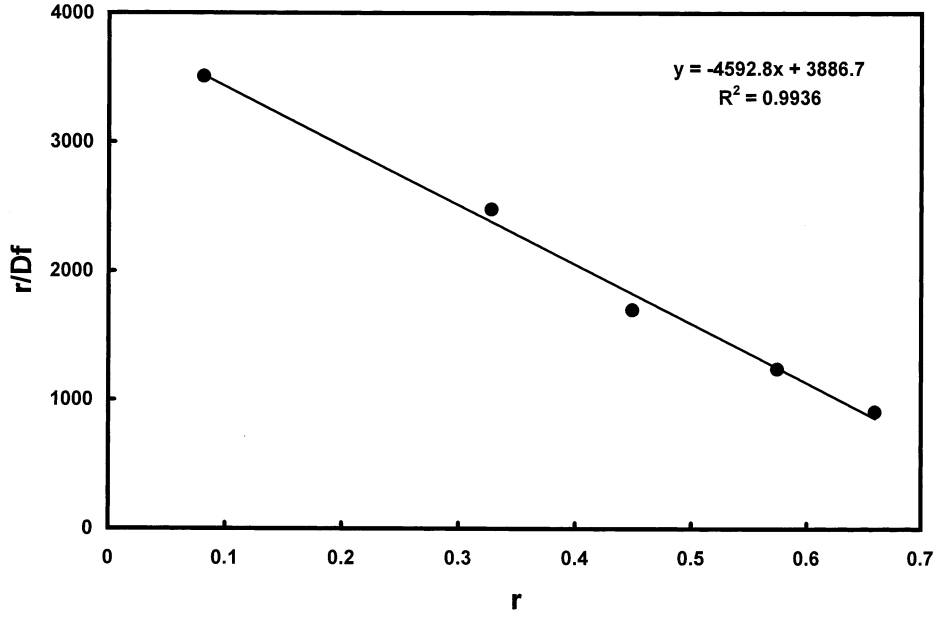

Fig. 1. Scatchard plot for 1:1 diflunisal:HPBCD binding. The points represent the experimental data (Determination 1) and the line represents the linear regression fit of the data.

Connors [18], an acceptable level of agreement between K_{11} (K_c) values for 1:1 complexation of substrates with cyclodextrins is that they are within a factor of ~ 2 from one another. In fact, Connors [18] refers only to binding to the 'parent' α, β and γ-cyclodextrin structures. In this study, and the one by Sideris et al. [9], the 2-hydroxypropyl derivatives of β-cyclodextrin were used. Since Sideris et al. [9] did not report the degree of substitution (D.S.) of their HPBCD sample, a factor which is capable of affecting the binding to the cyclodextrin [19], then the agreement of the dialysis data with the potentiometric data is very reasonable. The K_c determined by the potentiometric method [9] was reported to be 5564 ± 56 M^{-1}. Therefore the equilibrium dialysis method described here can be used reliably for studying drug binding to cyclodextrins. The potentiometric method is also a relatively simple method; however, it requires the use of ion-selective electrodes which are not always easy to construct. As previously discussed, there are several advantages of equilibrium dialysis over phase-solubility methods which can make it the preferred method.

The equilibrium dialysis method can be used in place of phase solubility or other methods for determining the stoichiometry of complexation. However, to ensure the reliability of any experimental method or the parameters derived from it, it is generally good practice to use more than one method of determination. The good agreement between the results of the equilibrium dialysis and titration microcalorimetry methods reported here with the previously reported data for diflunisal employing an ion-selective electrode [9] indicates the reliability of the equilibrium dialysis procedure.

The major factor limiting the widespread use of the equilibrium dialysis method is the availability of a dialysis membrane with an appropriate selectivity for the cyclodextrin and drug molecules to be studied. Ideally, the membrane should totally restrict the passage of the cyclodextrin but allow free movement (equilibration) of drug molecules. At the present time, there is a limited commercial availability of dialysis membranes with a low molecular weight cutoff (≤ 500 Da). The molecular weight cutoff of the Capran membrane is not

known; however, this membrane was used previously [12] to separate similar sized molecules and proved to be selectively permeable to HPBCD and diflunisal in this study. The molecular weight of diflunisal (MW = 250) is in the same MW range as other typical drug molecules, therefore the Capran membrane should be applicable to the study of binding of many other drugs to cyclodextrins. It is conceivable that as the use of the dialysis method becomes more widespread, the commercial availability of membranes with a low molecular weight cutoff will increase.

The linearity of the Scatchard plot indicates the formation of a 1:1 diflunisal:cyclodextrin complex. The 1:1 stoichiometry can be further verified by substituting free and bound drug, and ligand concentrations into the law of mass action expression for the complexation constant (K_c). For a 1:1 complex, the following equations based upon the law of mass action at equilibrium can be written:

$$D + L \overset{K_c}{\rightleftharpoons} DL \tag{5}$$

$$K_c = C_{DL}/(C_{Dt} - C_{DL})(n \cdot C_{Lt} - C_{DL}) \tag{6}$$

where C_{Dt}, C_{Lt}, C_{DL} and K_c are the total drug concentration, the total ligand concentration, the concentration of the complex (i.e. bound drug) and the complexation constant for a 1:1 complex, respectively. $C_{Dt} - C_{DL}$ and $n \cdot C_{Lt} - C_{DL}$ represent the unbound drug and unbound ligand concentrations, respectively. The K_c values estimated from the mass action law method were approximately 4000 (except for trial 4) and agreed quite well with those from the equilibrium dialysis and microcalorimetric methods. The constancy of K_c values calculated using Eq. (6) was verified for diflunisal:HPBCD binding (Table 2), indicating the formation of a 1:1 complex.

The thermodynamics of cyclodextrin binding is complex due to a number of driving forces which may be operative concurrently. In general, cyclodextrin binding processes are exothermic and exhibit a fairly large negative enthalpy change (ΔH) and an entropy change (ΔS) which can be either positive or negative. One fairly widely accepted mechanism involves the displacement of water molecules from the interior of the cyclodextrin cavity as a result of the inclusion of guest molecules within the cavity. The expulsion of this 'enthalpy-rich' water from the cavity is believed to be responsible for the negative ΔH values often associated with cyclodextrin binding. One possible explanation for the complexation of diflunisal with HPBCD involves the interaction of the aromatic ring of diflunisal with the cyclodextrin torus.

Table 2
Calculated complexation constants (K_c) for diflunisal–HPBCD binding using equilibrium dialysis data and D_f vs D_t fitted parameters

Mean D_t[a] (mg/ml)	K_c (M^{-1})			
	Trial 1	Trial 2	Trial 3	Trial 4
0.2	4367	4152	4117	3680
0.7	4452	4137	3935	3520
1.0	3903	3809	3771	3157
1.3	3993	4413	4277	3146
1.6	4036	4046	3980	3356
Mean	4150	4111	4016	3372
SD	243	217	191	231
%CV	5.9	5.3	4.8	6.9

[a] The K_c values were generated using the actual measured values of D_t for each trial.

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