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Fluorimetric Studies of the Formation of Riboflavin-β-Cyclodextrin Inclusion Complex: Determination of the Stability Constant by use of a Non-linear Least-squares Model

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Abstract

The non-linear least-squares model for calculation of the stability constant (K_{st}) of a drug-cyclodextrin complex has been used in fluorimetry studies.

Complexation of riboflavin with β-cyclodextrin (β-CyD) was monitored fluorimetrically by measuring changes in the fluorescence intensity of the vitamin in the presence of various amounts of β-CyD. Formation of an inclusion complex was confirmed in the solid state by differential scanning calorimetry (DSC) and in aqueous solution by proton nuclear magnetic resonance spectroscopy (^{1}H NMR).

The experimental K_{st} value (2112 M^{-1}) derived from the fluorimetry studies appeared to fit well to a 1 : 1 drug-to-cyclodextrin molar ratio according to the non-linear mathematical model. The model is particularly suitable for fluorescent compounds of which fluorescence intensity is influenced by the presence of cyclodextrins.

Cyclodextrins (CyDs) are cyclic doughnut-shaped oligosaccharides consisting of at least six 1 → 4-linked D-glucose units and having a cavity of fixed size and shape into which guest molecules can be accommodated (inclusion complexes) (Saenger 1980). An important prerequisite for the preparation and use of inclusion complexes is an understanding of the mechanism of association of the two species during complexation and, to that end, the stability constant (K_{st}) and stoichiometry of the complex must be known.

Determination of K_{st} can be performed by a number of linear procedures (Duchene 1987) but most of these suffer from theoretical and practical drawbacks (Djedaini & Perly 1993) including high concentrations of one of the interacting moieties, poor solubility of certain compounds, saturation binding (boundary condition) with respect to the ratio of the concentrations of the two binding partners and the occasional formation of dimers. On the other hand, non-linear procedures are free from solubility limitations (and thus have much broader applicability) (Diederich 1988) and are likely to displace those based on the Benesi–Hildebrand, Scott or Scatchard linear models (Diederich 1988).

Non-linear models can be generally worked out in three different ways (Draper & Smith 1981): linearization (or Taylor series), the steepest descent method, and non-linear regression by minimizing the squared residuals of the relevant parameters. Several algorithms are available for the analysis of non-linear mathematical models (Fox 1984) by non-linear regression, with the Levenberg–Marquardt algorithm being one of the most commonly used for the analysis of unconstrained models (Marquardt 1963). Non-linear mathematical models have recently been described for the calculation of K_{st} in methods employing potentiometry (Sideris et al 1992) and UV spectrophotometry (Rose & Drago 1959) during complexation, or nuclear magnetic resonance (NMR; Djedaini et al 1990). In addition, the Gauss–Newton non-linear optimization method has been used for the analysis of solubility curves (Miyahara & Takahashi 1982) and the Marquardt algorithm for the correlation of retention data of the guest molecule in the presence of CyD with the K_{st} (Spino & Amstrong 1987) using HPLC.

In this work we have applied non-linear curve-fitting models for the calculation of K_{st} on the basis of changes in the fluorescence intensity of riboflavin in the presence of β-cyclodextrin (β-CyD).

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Theoretical

Fluorimetry can be used for the calculation of K_{st} in a variety of ways, including changes in fluorescence polarization (Bright et al 1985). Such changes reflect the relaxation time of molecules, thus providing direct evidence for complex formation and reduction (through quenching) in fluorescence intensity of the guest molecule on inclusion in the cyclodextrin cavity (Connors 1987). As in some cases inclusion reactions might be slow, it is essential that equilibrium (indicated by a fluorescence reading that remains unchanged with time) is reached before the measurement of fluorescence intensity. In this respect, the linear models (Connors 1987) employed for the calculation of K_{st} by correlating fluorescence intensity with the cyclodextrin concentration use excess amounts of cyclodextrin and make the assumption that free CyD is equal to total CyD (CyD_t). The system described below avoids such an assumption.

Consider a system with the guest (G) riboflavin and the cyclodextrin (β-CyD; shown as CyD in the equations below) at total concentrations of G_t and CyD_t, respectively. In the absence of CyD the fluorescence intensity of G_t is:

$$F_0 = k_G G_t \quad (1)$$

where k_G is the proportionality constant correlating the guest concentration with the fluorescence intensity. By adding to this system the β-CyD solution and considering that the fluorescence intensity is the sum of contributions, we write:

$$F = k_G[G] + k_{1:1}[CyD\text{-}G] + k_{CyD}[CyD] \quad (2)$$

where $k_{1:1}$ and k_{CyD} are the proportionality constants for the 1 : 1 inclusion complex and pure cyclodextrin, respectively. As CyDs do not fluoresce, their contribution to fluorescence is zero and, therefore, $k_{CyD}[CyD]$ is also zero. Equation 2 can be thus rewritten:

$$F = k_G[G] + k_{1:1}[CyD\text{-}G] \quad (3)$$

The concentration of the complex can be calculated from equation 3 as:

$$[CyD\text{-}G] = (F - k_G[G])/k_{1:1} \quad (4)$$

The observed difference in fluorescence (ΔF) is derived from equations 1 and 3:

$$\Delta F = F_0 - F = k_G[G_t] - (k_G[G] + k_{1:1}[CyD\text{-}G])$$

which, by transformation, gives:

$$\Delta F = \Delta k[CyD\text{-}G] \quad (5)$$

where $\Delta k = k_G - k_{1:1}$.

From the kinetic equilibrium during complex formation ($[CyD\text{-}G] = K_{st}[G][CyD]$) and the mass balance for the cyclodextrin ($[CyD] = [CyD_t] - [CyD\text{-}G]$), the concentration of the complex [CyD-G] can be calculated from:

$$[CyD\text{-}G] = [CyD_t]K_{st}[G]/(1 + K_{st}[G]) \quad (6)$$

Substitution of [CyD-G] from equation 6 into equation 5 gives:

$$\Delta F = \Delta k[CyD_t]K_{st}[G]/(1 + K_{st}[G]) \quad (7)$$

Furthermore, by applying the mass balance to the guest concentration ($[G] = [G_t] - [CyD\text{-}G]$) and substituting the complex concentration from equation 4, we can write:

$$[G] = (k_{1:1}[G_t] - F)/(k_G - k_{1:1})$$

Also, substitution of $k_{1:1}$ with its equivalent, $(k_G - \Delta k)$, in the numerator and of $k_G - k_{1:1}$ with its equivalent, Δk, in the denominator of the equation above, gives:

$$[G] = -((k_G - \Delta k)[G_t] - F)/\Delta k$$
$$[G] = -(k_G[G_t] - \Delta k[G_t] - F)/\Delta k$$

Moreover, substitution of $k_G[G_t]$ with its equivalent, F_0, gives:

$$[G] = ((F_0 - F) - \Delta k[G_t])/\Delta k$$
$$[G] = (\Delta k[G_t] - \Delta F)/\Delta k \quad (8)$$

Finally, substitution of [G] from equation 8 into equation 7, gives:

$$\Delta F = \Delta k[CyD_t]\{K_{st}(\Delta k[G_t] - \Delta F/\Delta K\}/$$
$$\{1 + k_{st}(\Delta k[G_t] - \Delta F)/\Delta k\}$$

which, by simple transformation, gives:

$$\Delta F = \Delta k[CyD_t]\{K_{st}(\Delta k[G_t] - \Delta F)\}/$$
$$\{\Delta k + K_{st}(\Delta k[G_t] - \Delta F)\} \quad (9)$$

Equation 9 is a model without approximations, correlating the observed differences in fluorescence intensity (ΔF) with the initial total concentrations of the guest $[G_t]$ and cyclodextrin $[CyD_t]$. The unknown parameters K_{st} and Δk can be calculated from this model using non-linear least-squares regression. By substituting [G] in equation 6 with its equivalent in equation 8, the concentration of the complex [CyD-G] can, moreover, be calculated at any combination of the total concentrations of cyclodextrin and guest.

Materials and Methods

Materials

Riboflavin-5′(dihydrogen-phosphate monosodium salt) was purchased from Aldrich (Gillingham, Dorset, UK) and β-cyclodextrin from Janssen (Beerse, Belgium). Deuterium oxide (99·9%) was obtained from Fluka (Poole, Dorset, UK). Double-distilled water was used throughout. All other reagents were of analytical grade.

Instrumentation

The fluorescence intensity of riboflavin in the presence of β-CyD was monitored by use of a Perkin-Elmer LS-3 fluorescence spectrophotometer (excitation and emission wavelengths of 445 and 520 nm, respectively).

Characterization of the riboflavin-β-CyD complex in the solid state was performed by differential scanning calorimetry (DSC); thermograms were obtained by use of a Perkin-Elmer DSC 7 instrument using vented aluminium pans. Typical conditions were: temperature range, 50–300°C; scanning rate, 10° min^{-1}; sample weight, 10 mg. Baseline optimization was performed before each run. Characterization of the riboflavin-β-CyD complex in aqueous solutions was performed by ^1H NMR. ^1H NMR spectra in D_2O were recorded on a Bruker AM 500 spectrometer connected to an Aspect 3000 computer. The chemical shifts were related to the residual solvent signal (HDO = 4·84 ppm at 293 K). Typical conditions were 16 k data points with zero filling, sweep width 5 kHz giving a digital resolution of 0·61 Hz $point^{-1}$, pulse width 4 μs, acquisition time 1·64 s and number of scans 64.

Statgraphics Plus version 6 (Manugistics, Rockville, MD, USA) was used for the non-linear calculations and for the illustration of graphics.

Preparation of the riboflavin-β-CyD inclusion complex

The inclusion complex of riboflavin with β-CyD was prepared by the freeze-drying method (Loukas et al, 1995). Riboflavin (0·5 mmol) was dissolved in distilled water (10 mL) and the clear solution was added dropwise to a solution of β-CyD (0·5 mmol) in water (10 mL) with stirring. The mixture was stirred in the dark at 20°C for two days. The final clear yellow solution was freeze-dried to yield amorphous yellow powder which was characterized in the solid state by DSC and in the aqueous state by ^1H NMR (see above).

Results and Discussion

Characterization of the riboflavin-β-CyD inclusion complex

Results (Fig. 1) from DSC of the riboflavin-β-CyD complex are consistent with true complexation. In the thermogram of free riboflavin (Fig. 1c) there are two peaks, one exothermic at 256°C and one endothermic at 279°C; these probably correspond to the crystallization and melting points of the vitamin,

respectively. These two peaks disappear, however, in the thermograms obtained from the complex (Fig. 1b) and a new peak appears at 293·5°C; this presumably corresponds to the thermal dissociation of the complex.

Observed resonances in the NMR study of the soluble riboflavin-β-CyD complex were the time-averaged peaks obtained from β-CyD, riboflavin and its inclusion complex (fast exchange regime on the NMR time-scale at 293 K). The formation of the riboflavin-β-CyD complex in aqueous solution is evidenced by the modification of the NMR spectrum of riboflavin. Fig. 2, the 500 MHz NMR spectra of the complex and the free compounds, reveals that under the conditions employed only shift changes of the signal occurred. There were no new peaks that could be assigned to the complex itself, an observation suggesting that the complexation of riboflavin with β-CyD is a dynamic process with the riboflavin being in a state of fast exchange (relative to the NMR time-scale) between the free and included forms at a rate which must exceed the reciprocal of the largest observed shift difference (in Hz) for any proton of the riboflavin molecule (Djedaini et al 1990).

Results (Table 1) from the NMR study show that the internal protons (H3 and H5) of β-CyD undergo greater riboflavin-induced chemical shift changes than the external H1, H2, H4 and H6 protons, indicating that the vitamin has approached the cavity. Although of low magnitude, the H3 and H5 shifts observed are nevertheless indicative of the occurrence of inclusion (Komiyama 1989), especially as δ values for the external protons remained practically unchanged (Table 1). Digital integration of the NMR signals of the phenyl protons of riboflavin (H6′–H9′; Fig. 2b) and of the anomeric protons of β-CyD (H1′; Fig. 2a) in the complex, enabled direct estimation of the stoichiometry coefficient. This was found to be approximately 1 : 1, implying that all of the riboflavin molecules are in the complexed form; two different signals for the riboflavin phenyl protons of the free and complexed riboflavin would have been expected if some of the riboflavin molecules were in the free form.

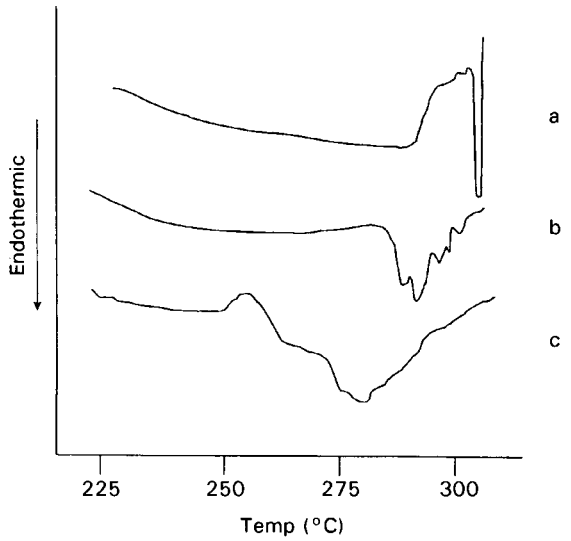

FIG. 1. DSC thermograms of a, β-CyD, b, riboflavin-β-CyD inclusion complex (c) riboflavin.

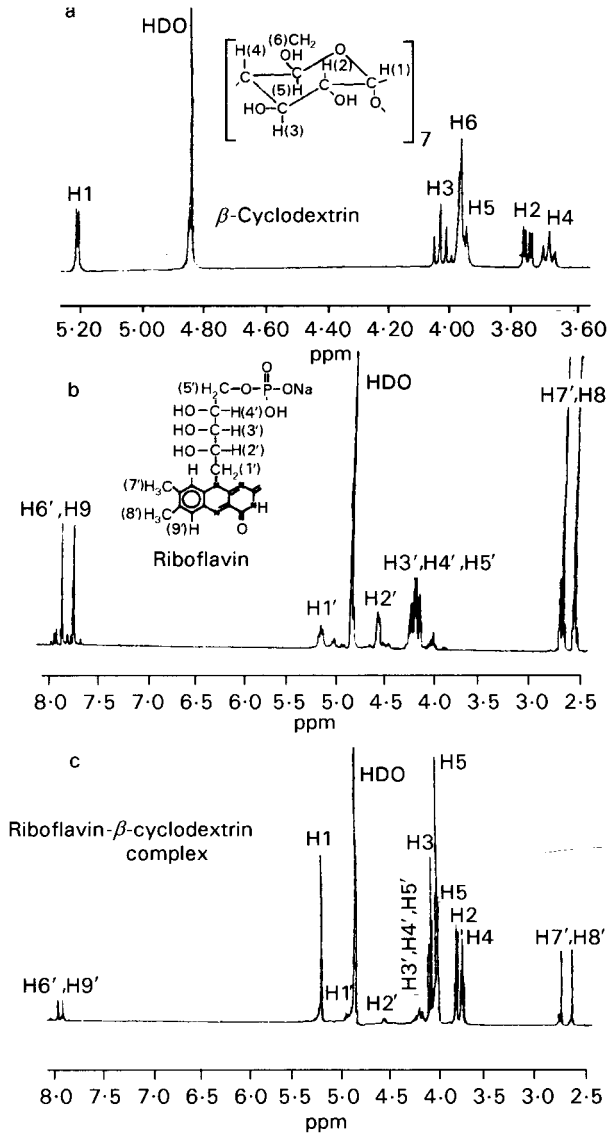

FIG. 2. 500 MHz ^{1}H NMR spectra of a, β-CyD, b, riboflavin, c, riboflavin-β-CyD inclusion complex. Numbers in parentheses denote protons with chemical shifts corresponding to numbered peaks in the spectra.

Calculation of the stability constant K_{st}

As already alluded to, the fluorescence intensity, F, of the free riboflavin changes in the presence of β-CyD. This difference in fluorescence intensity (ΔF) of riboflavin in relation to the concentration of CyD is used for the calculation of K_{st} (equation 9). In order to calculate the starting values for the iteration procedure, the K_{st} (2653 M^{-1}; estimated from the kinetic model by monitoring the photodegradation of riboflavin in the presence of increasing concentrations of β-CyD, results not shown) and a value for ΔF (corresponding to the presence of a specific β-CyD concentration) were substituted in equation 9. The value of Δk was calculated by solving a simple quadratic equation:

$$(a^2 + bx + c = 0;\ x_{1,2} = \{-b \pm \sqrt{(b^2 - 4ac)}\}/2a) \quad (10)$$

The graphical solution of equation 9 is given in Fig. 3. In this model the iteration routine ends when the relative change in

Table 1. NMR chemical shifts of the protons of β-CyD and riboflavin in the free states and in the inclusion complex.

Proton	δ_0 (free)	δ_C (complex)	$\Delta\delta$ ($=\delta_C - \delta_0$)*
β-CyD			
H1	5·205	5·206	0·001
H2	3·751	3·750	−0·001
H3	4·031	4·020	−0·011
H4	3·683	3·685	0·002
H5	3·949	3·940	−0·009
H6	3·964	3·966	0·002
Riboflavin			
H6′,H9′	7·900	7·980	0·080
H6′,H9′	7·770	7·930	0·160
H7′,H8′	2·620	2·650	0·030
H7′,H8′	2·496	2·540	0·044
H1′	5·133	5·136	0·003
H2′	4·513	4·516	0·003
H3′,H5′	4·149	4·146	−0·003
H3′,H5′	4·160	4·158	−0·002

*Negative values indicate up-field movement (shielding effect).

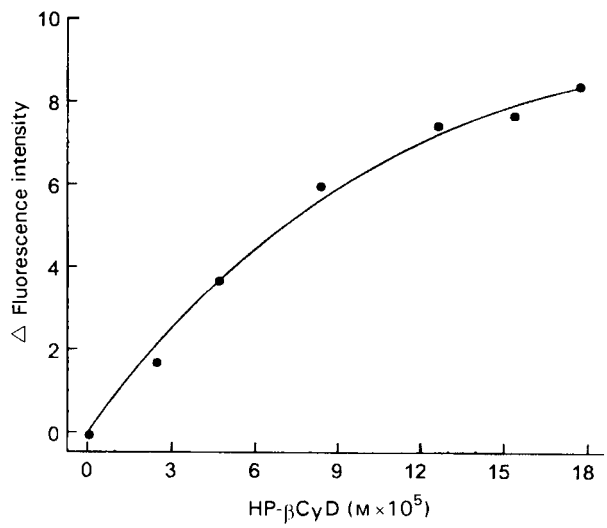

FIG. 3. Plot of the observed ΔF values for riboflavin and the estimated fitting line according to equation 9.

the residual sum of squares between iteration is less than or equal to the convergence criterion. The starting values for Δk and K_{st} result in a value of 2112 ± 193 M^{-1} for K_{st} and, judging from the r^2 value (0·975), the model appears to fit the observed value well. It thus appears that different methods (kinetic and fluorimetric studies) give different (albeit slightly) K_{st} values. Such differences in K_{st} values are commonly observed in studies where the K_{st} is calculated by different methods. For example, the K_{st} of the complex of *p*-nitrophenol with β-CyD (1 : 1 molar ratio) is 126 M^{-1} and 250 M^{-1} when calculated by titration calorimetry and spectrophotometry respectively (Rosenske & Connors 1980), and the K_{st} of the complex of *p*-nitrophenolate with β-CyD has values of 1590 M^{-1} when measured by optical rotation (Bergeron et al 1977) and 3550 M^{-1} when measured by gel filtration (Korpela & Himanen 1984).

In conclusion, this work has shown that the non-linear curve fitting model described can be used for the calculation of the K_{st} of drug-cyclodextrin inclusion complexes. As these models require only the values of initial concentrations of the free species (drug and CyD) without any limitation (e.g. an excess amount of one of the two species), experimental and theoretical drawbacks are avoided. This model is particularly suitable for fluorescent compounds where fluorescence intensity is influenced by the presence of cyclodextrins.

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