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Measurement of Molecular Association in Drug : Cyclodextrin Inclusion Complexes with Improved ^{1}H NMR Studies

YANNIS L. LOUKAS

Centre for Drug-Delivery Research, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK

Abstract

The molecular association of haloperidol with hydroxypropyl-β-cyclodextrin, expressed by the binding constant of the inclusion complex formed, was calculated from the changes on the ^{1}H NMR spectra of the drug in the presence of the cyclodextrin.

The stoichiometry of the complex was calculated by use of the continuous variation method (Job plot), and found to be 1:1. The binding constant for the 1:1 complex was calculated using improved non-linear models, which were solved by non-linear least-squares regression analysis, applying an iteration procedure.

Three improved mathematical models for more accurate calculation of binding constants are proposed. The models are free from any assumptions and from practical or theoretical shortcomings.

Because haloperidol, an anti-depressant drug, has limited solubility in water, which limits its use, new non-acidic and water-soluble formulations of haloperidol have been proposed on the basis of the complexation of the drug with a variety of cyclodextrin derivatives (Loukas 1995). Cyclodextrins are cyclic oligosaccharides composed of D-glucopyranose units linked α-1,4. They are known to form non-covalent water-soluble inclusion complexes with a wide variety of drugs, thus improving their solubility, stability or bioavailability. Because of increasing interest in cyclodextrins and their inherent usefulness, a variety of studies has been conducted to clarify the mechanism of complexation and to calculate the binding constant and stoichiometries of the complexes formed.

Various methods for the determination of the binding constant have being described in the literature (Saenger 1980); these are based on techniques such as conductometric titrations, potentiometric and spectrophotometric methods, solubility studies and competitive indicator binding. These methods are mostly based on graphical solutions using linear least-squares regression analysis applied to known mathematical models (Benesi–Hildebrand, Scatchard, etc). Most of these models employ assumptions, for example they assume the concentrations of the interacting species and products, poor solubility of certain compounds, a boundary condition (saturation binding) in respect of the ratio of the concentrations of the two binding partners and the occasional formation of dimers; these assumptions have theoretical and practical drawbacks (Djedaini & Perly 1993). It has been suggested (Schneider et al 1984; Ferguson & Diederich 1986; Diederich 1988) that non-linear procedures, being free from the above-mentioned assumptions, have much broader applicability and are likely to displace evaluations performed by use of linear models. Non-linear mathematical expressions have already been described for calculation of binding constants using, for instance, solubility curves (Miyahara & Takahashi 1982) and HPLC methods (Armstrong et al 1986). Recent efforts in the field include the fluorimetric (Loukas 1997a) and kinetic (Loukas 1997b) determination of binding constants by use of improved non-linear mathematical models.

In this work, the molecular association between haloperidol and hydroxypropyl-β-cyclodextrin (HPβCyD) and the stoichiometry of the complex formed were examined by ^{1}H NMR studies in linear models and the resulting values were evaluated using improved non-linear models.

Theory

^{1}H NMR studies

During complexation the chemical environment of some protons changes; this results in changes in the chemical shifts of the ^{1}H NMR lines of the protons (shielding or deshielding effects). Specifically, the cyclodextrin internal protons (H-3 and H-5) and the protons of the guest (G, haloperidol in this study) included in the hydrophobic cyclodextrin cavity are the most affected.

Consider the typical 1:1 equilibrium between haloperidol (G) and HPβCyD (CyD). Their total concentrations are denoted as G_t and CyD_t respectively. The fractions of the free (f_0) and the complexed haloperidol (f_1) in such an equilibrium, are expressed:

$$\begin{aligned} f_0 &= G/G_t = G/(G + G{:}CyD) \\ &= G/(G + K_{1:1}G\ CyD) = 1/(1 + K_{1:1}CyD) \end{aligned} \quad (1)$$

$$\begin{aligned} f_1 &= (G{:}CyD)/G_t = K_{1:1}G\ CyD/(G + G{:}CyD) \\ &= K_{1:1}G\ CyD/(G + K_{1:1}G\ CyD) \\ &= K_{1:1}\ CyD/(1 + K_{1:1}CyD) \end{aligned} \quad (2)$$

and

$$f_0 + f_1 = 1 \quad (3)$$

where $K_{1:1}$ is the binding (equilibrium) constant and G and CyD are the concentrations of the uncomplexed (free) haloperidol and HPβCyD, respectively. The chemical shift (δ) of

Present address: Y. L. Loukas, Riga Ferreou 21, Ano Ilioupolis, 163 43 Athens, Greece.

haloperidol protons is a weighted average of the shift attributed to the uncomplexed (δ_G) and that attributed to the complexed haloperidol ($\delta_{G\text{-}CyD}$). Thus, can be

$$\delta = f_0\delta_G + f_1\delta_{G:CyD} \quad (4)$$

Substitution of equation 3 into equation 4 gives sequentially:

$$\delta - \delta_G = f_1(\delta_{G:CyD} - \delta_G) \Leftrightarrow \Delta\delta_{obs} = f_1\Delta\delta_c \quad (5)$$

Substitution of f_1 from equation 2 into equation 5 results in the expression:

$$\delta_{obs} = [K_{1:1}CyD/(1 + K_{1:1}CyD)]\Delta\delta_c \quad (6)$$

To solve equation 6 the concentration CyD is needed. At this point, the assumption that $CyD = CyD_t$ is employed (use of a high excess of CyD_t compared with G_t) and through this, equation 6 is transformed into a double reciprocal linear equation (Loukas et al 1995) (equation 7), known as a Benesi–Hildebrand equation.

$$1/\Delta\delta_{obs} = [1/(K_{1:1}\Delta\delta_c CyD_t)] + 1/\Delta\delta_c \quad (7)$$

To avoid this assumption, three different processes followed for the NMR study led to non-linear models.

Firstly, further substitution of $f_1 = G{:}CyD/G_t$ in equation 5, results in:

$$G{:}CyD = \Delta\delta_{obs}G_t/\Delta\delta_c \quad (8)$$

Substitution of the quantity G-CyD from equation 8 into the cyclodextrin mass balance equation gives:

$$CyD = CyD_t{:}G - CyD = CyD_t - (\Delta\delta_{obs}G_t/\Delta\delta_c) \quad (9)$$

Finally, substitution of equation 9 into equation 6 gives, after transformation:

$$\Delta\delta_{obs} = \{K_{1:1}(\Delta\delta_c CyD_t - \Delta\delta_{obs}G_t)/ [\Delta\delta_c + K_{1:1}(\Delta\delta_c CyD_t - \Delta\delta_{obs}G_t)]\}\Delta\delta_c \quad (10)$$

Equation 10 involves no approximations and correlates the initial total concentrations of the guest and the cyclodextrin (G_t and CyD_t) with the observed differences in chemical shift ($\Delta\delta_{obs}$). The unknown parameter $K_{1:1}$ can be then calculated according to equation 10, using an iteration procedure (discussed below).

Secondly, use of the mass balance equations for the guest and the cyclodextrin and the main equation for the binding constant gives, after transformation, a quadratic equation for CyD (Appendix). This correlates the initial concentration of the guest and the cyclodextrin with $K_{1:1}$. Substitution of CyD from equation A2 into equation 6 gives:

$$\Delta\delta_{obs} = [\Delta\delta_c\{-(K_{1:1}G_t - K_{1:1}CyD_t + 1) \pm \sqrt{(K_{1:1}G_t - K_{1:1}CyD_t + 1)^2 + 4K_{1:1}CyD_t}\}/ [2 - (K_{1:1}G_t - K_{1:1}CyD_t + 1) \pm \sqrt{\{(K_{1:1}G_t - K_{1:1}CyD_t + 1)^2 + 4K_{1:1}CyD_t\}}] \quad (11)$$

Equation 11 correlates the absorbance differences ($\Delta\delta_{obs}$) with the initial total concentrations G_t and CyD_t, without any approximations. Equation 11 is solved iteratively and the erroneous solution (either negative or redundant) is discarded.

Thirdly, defining the average number of cyclodextrin molecules bound per guest molecule as $\bar{n}$ gives the expression:

$$\bar{n} = \sum(\text{CyD bound to G})/\sum(\text{all G}) \quad (12)$$

Further definition of the quantity Σ(CyD bound to G) as CyD_b, the 'total bound' cyclodextrin concentration, can be written:

$$CyD_b = G{:}CyD \quad (13)$$

The free cyclodextrin concentration, CyD, is obtained from:

$$CD = CD_t - \bar{n}G_t \quad (14)$$

Using the term n and substituting the quantity CyD in equation 6 with its equivalent from equation 14, gives:

$$\Delta\delta_{obs} = \frac{K_{1:1}(CyD_t - \bar{n}G_t)}{1 + K_{1:1}(CyD_t - \bar{n}G_t)}\Delta\delta_c \quad (15)$$

To solve equation 15, $\bar{n}$ is needed; this is derived from the continuous variation method (Connors 1987) (discussed below).

Materials and Methods

Materials

Haloperidol was obtained from Sigma (Poole, Dorset, UK) and HPβCyD from Wacker Chemie (Munich, Germany). HPβCyD has a degree of substitution (DS) of 0·4 (number of hydroxypropyl groups per unit of anhydroglucose) and a relative molecular mass (M_r) of 1300. The DS value (a measure of the extent to which the reactive hydroxyl groups in each glucose unit of the ring have been substituted) obtained by digital integration was confirmed by the ^{1}H NMR spectrum of HPβCyD in deuterium oxide. Deuterium chloride (DCl) and deuterium oxide (D_2O) were purchased from Fluka (Poole, Dorset, UK); double distilled water was obtained from a MilliQ system (Waters). Other reagents were of analytical grade.

Instrumentation

Characterization of the haloperidol-HPβCyD complex in aqueous solutions was performed by ^{1}H NMR spectroscopy. Spectra were obtained in 10% DCl and recorded on a Brucker AM 500 spectrometer connected to an Aspect 3000 computer. Chemical shifts were measured relative to the residual solvent signal (hydrogen-deuterium chloride = 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 128 for the complexes and 640 for pure haloperidol (because of its low solubility).

Continuous variation method (Job plot)

For a system containing a 1:1 (G:CyD) complex, the quantity $\bar{n}$ ($= CyD_b/G_t$) can range from 0 to 1 ($0 < \bar{n} < 1$). Thus, $\bar{n}$ is a useful indication of the extent of the binding isotherm that has been examined. In a 1:1 system, $\bar{n}$ is equal to $f_{1:1}$, the fraction of the guest present as the 1:1 complex. In this study the stoichiometry was calculated by using the continuous variation plot and the corresponding value for $\bar{n}$ was used in equation 15. The continuous variation technique (Job plot) was based on the difference in chemical shift ($\Delta\delta = \delta_0 - \delta$) of haloperidol

observed in the presence of HPβCyD. Equimolar solutions of haloperidol and HPβCyD were prepared and mixed to standard volume and proportions such that the total concentration remained constant ($G_t + CyD_t = M$).

$\Delta\delta$ values for the haloperidol preparations were calculated by measuring the chemical shift of haloperidol in the absence (δ_0) and presence (δ) of HPβCyD. Subsequently, $\Delta\delta G_t$ was plotted against r ($= G_t/[G_t + CyD_t]$). The concentrations of the free guest and cyclodextrin in a 1:n inclusion complex G:CyD_n can then be expressed:

$$G = rM - G{:}CyD$$
$$CyD = M(1 - r) - nG{:}CyD$$

For a given value of r the concentration of the complex G:CyD will reach a maximum corresponding to the point where the derivative $d(G{:}CyD)/dr = 0$. Differentiation of the above two equations with respect to r gives $dG/dr = M$ and $dCyD/dr = -M$. Rearrangement of the above equations leads to a single solution: the maximum absolute complex concentration is reached for $r = (n+1)^{-1}$ and does not depend on M or on the binding constant.

Results and Discussion

Determination of the complex stoichiometry

The stoichiometry of the complex must be calculated before proceeding with any binding-constant calculations. Determination of the stoichiometry of the haloperidol–HPβCyD complex, by the continuous variation method, was based on ^{1}H NMR spectra obtained for haloperidol and HPβCyD mixtures in which the total initial concentrations of the two species were maintained constant and the ratio (r) of the initial concentrations varied between 0 and 1 (see Materials and Methods). If a physical parameter directly related to the concentration of the complex (for instance the chemical shift, δ) can be measured under these conditions, and is plotted as a function of r, the maximum value for this parameter will occur at $r = m/(m+n)$, where m and n are, respectively, the proportions of haloperidol and HPβCyD in the complex $G_m{:}CyD_n$. For instance, if the stoichiometry of the complex is 1:1 ($m = n = 1$), the maximum value for the examined parameter will be reached at $r = 0{\cdot}5$; if the stoichiometry of the complex is 1:2 ($m = 1$, $n = 2$) the maximum value will be reached at $r = 0{\cdot}33$.

The calculated quantities $\Delta\delta G_t$ are proportional to the concentration of the complex, and can be plotted against r. The resulting continuous variation plots demonstrate that because r has a maximum value of almost 0·5 for haloperidol–HPβCyD (Fig. 1), the complex has 1:1 stoichiometry.

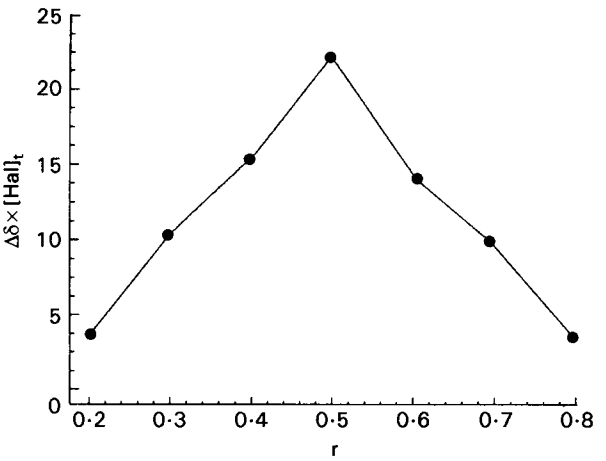

FIG. 1. Continuous variation plot (Job plot) for the haloperidol–HPβCyD complex.

Calculation and evaluation of the binding constant

The binding constant for the haloperidol–HPβCyD complex was calculated by using the mathematical models described above. In the ^{1}H NMR studies the observed resonances for the water-soluble complex were the time-averaged peaks obtained for the free HPβCyD and for haloperidol and their inclusion complex (fast-exchange regime on the NMR time-scale at 293 K). Formation of the inclusion complex in aqueous solution is evidenced basically by the modification of the ^{1}H NMR spectrum of haloperidol (Fig. 2). As haloperidol contains two phenyl groups connected by a short carbon chain, a stoichiometry of 1:2 is expected. However, results from the NMR study suggest that only the *p*-chlorobenzoyl group is involved in the inclusion process. This is supported by the findings (Fig. 2) that the haloperidol phenyl group with the attached fluorine atom seems to remain outside the cavity, as it does not undergo any significant spectral changes. These results therefore indicate the formation of a 1:1 inclusion complex and support the stoichiometry calculated by the Job-plot method. Failure of the fluorinated phenyl group to enter the cyclodextrin cavity is probably because of its polar nature, which renders the process of complexation unfavourable.

The ^{1}H NMR spectrum of haloperidol phenyl groups in free and complexed form obtained under the present conditions shows only shift changes of the corresponding signals. As there are no new peaks that could be assigned to the complex as such, complexation of haloperidol with HPβCyD appears to be a dynamic process with haloperidol being in a state of fast exchange (on the ^{1}H NMR time-scale) between the free and the included form. Thus, the exchange rate must exceed the reciprocal of the largest observed shift difference (in Hz) for any proton of the guest molecule.

Non-linear estimation procedure

The non-linear estimation of the parameters $K_{1:1}$ and $\bar{n}$ is based on an iteration procedure following specific algorithms. The proposed mathematical models were examined with different algorithms to observe any possible deviation of the calculated values. For instance, the Marquardt method is an iterative algorithm which at each iteration process evaluates the estimates against a set of control criteria. If successive iterations fail to change the sum of squares of the convergence criterion, the procedure stops. Furthermore, sequential quadratic programming was used; this is a doubly iterative algorithm. Briefly, each major iteration sets up a quadratic programme to determine the direction of search and the loss function is evaluated at any iterative point, until the search converges.

In standard multiple regression we estimate the regression coefficients by 'finding' those coefficients that minimize the residual variance (sum of squared residuals) around the regression line. Any deviation of an observed score from a predicted score signifies some loss in the accuracy of our

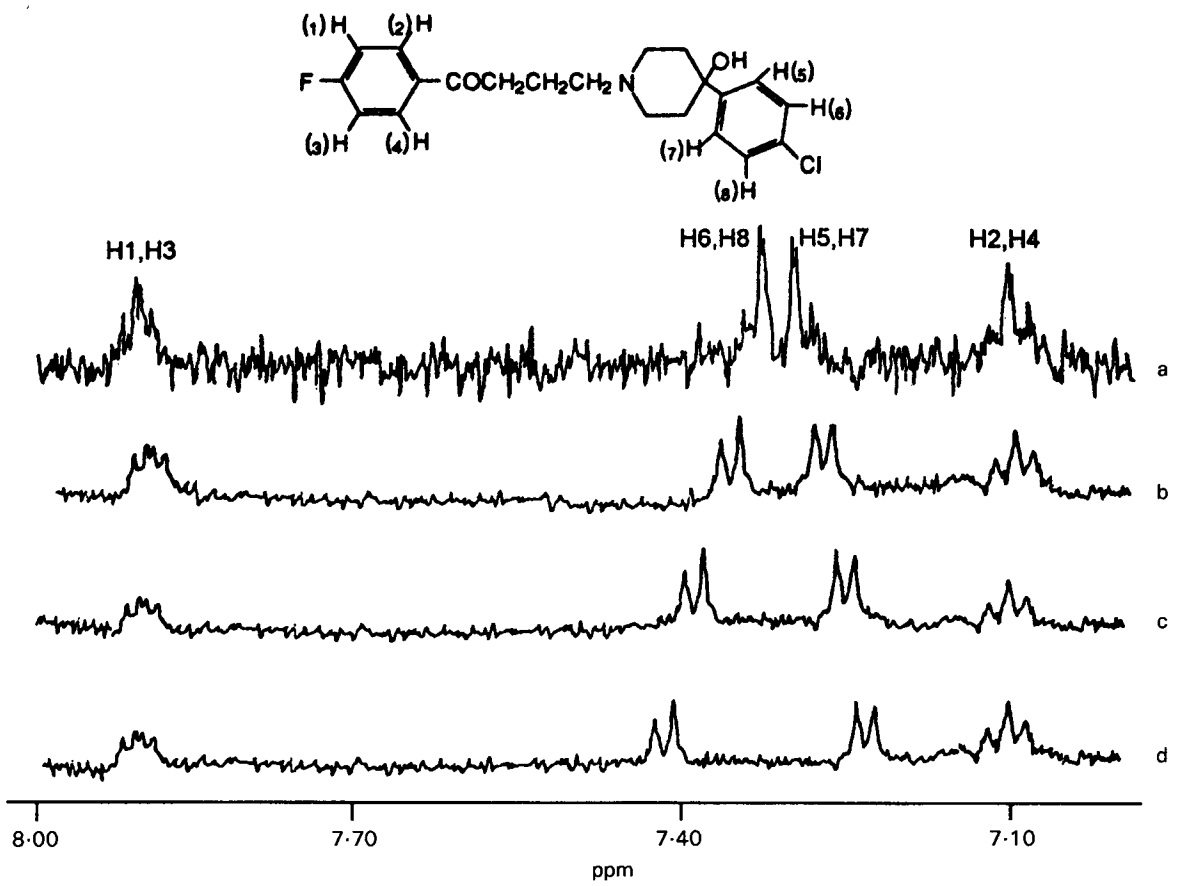

FIG. 2. Partial 500 MHz ^{1}H NMR spectra of haloperidol–HPβCyD mixtures. a. 10 mM haloperidol, b. 8 mM haloperidol and 2 mM HPβCyD, c. 6 mM haloperidol and 4 mM HPβCyD, d. 5 mM haloperidol and 5 mM HPβCyD.

prediction, for example, as a result of random noise (error). Therefore, we can say that the goal of the least-squares estimation is to minimize a loss function; specifically, this loss function is defined as the sum of the squared deviation about the predicted values. To minimize the loss function (to find the best-fitting set of parameters) and to estimate the standard errors of the parameters estimates, a very efficient algorithm was used (quasi-Newton) that approximates the second-order derivatives of the loss function and guides the search for the minimum.

The unknown parameters $K_{1:1}$ and $\bar{n}$ were calculated by use of the above mentioned non-linear procedure. For the iteration procedure to begin, starting values for the parameters must be defined. For instance, the starting value for $K_{1:1}$ was set at 100 (M^{-1}) and $\bar{n}$ was set at 0·1. The sequential quadratic programming method gives the opportunity to constrain any of the parameters. For instance, $\bar{n}$ can be constrained to be less than 1 ($\bar{n} < 1$) because the complex has a 1:1 stoichiometry. If the value of $\bar{n}$ exceeds 1, this means either that a higher-order complex is present (i.e., for 1:2 complexes $\bar{n} < 2$) or that there is a computational error. In the event of a computational error the stoichiometry of the complex could be examined by methods such as the continuous variation method (see above). The parameter $\bar{n}$ can give an idea of the extent of complexation and it could be characterized as 'the degree of complexation'. It is known (Schneider et al 1988) that only a percent of the guest molecules is involved in the complexation procedure. For instance, in the current case where $\bar{n} = 0{\cdot}79$ (Table 1), it could be concluded that 79% of the guest is in the complexed form and the rest, 21%, in the free form. The extent of complexation is an important factor, especially in therapeutics, where the pharmacological effect of a drug is directly related to its 'nature' (meaning in free or complexed form). For instance, complexation of indomethacin with β-cyclodextrin does not eliminate the side-effect of the drug because 40% of it is in free form (Djedaini et al 1990).

By comparing the three different models, it could be concluded that all gave comparable values for $K_{1:1}$ and that the third model (equation 15) resulted in the highest Rsq (Table 1). Because equation 15 calculates the parameters $K_{1:1}$ and $\bar{n}$ simultaneously and gives the highest Rsq value, it might be preferred to the other two models. Fig. 3 shows the graphical fitting of equation 15 to the experimental data. The deviations appearing in the values in Table 1 are acceptable; this is a common phenomenon in the literature. It should be mentioned that for a series of similar experiments (same guest with dif-

Table 1. Estimated values for the parameters $K_{1:1}$ and $\bar{n}$, from the non-linear models described.

Non-linear model	$K_{1:1}$	(M^{-1})	$\bar{n}$	Rsq
Equation 10	1412	(225)	–	0·823
Equation 11	1144	(167)	–	0·852
Equation 15	1711	(392)	0·79 (0·16)	0·946

Numbers in parentheses denote standard error.

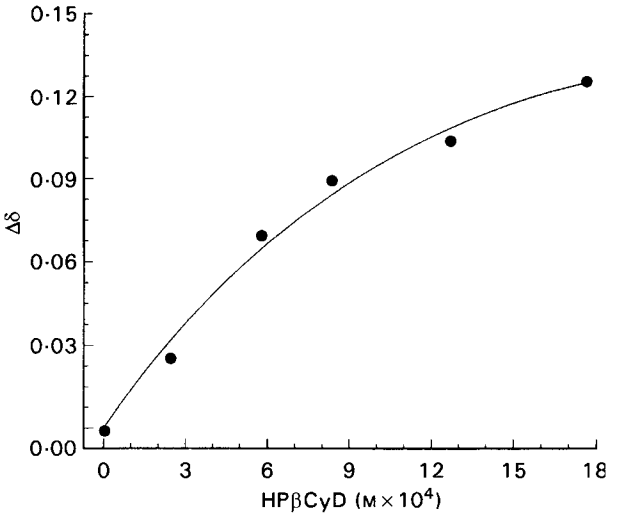

FIG. 3. Curve fitting of equation 15 to the experimental data.

ferent cyclodextrins), the same mathematical model must be used if the calculated parameters are to be comparable. The calculated values are also in accordance with the value of the binding constant for haloperidol–HPβCyD (2112 M^{-1}), calculated from the phase-solubility studies (Loukas et al 1997).

The significant deviation in binding constant values obtained from models solved by following a graphical and iteration procedure has been criticized in the literature. Linear transformation of the rectangular hyperbola type of binding constant is valid only when one of the two species is present in large excess. It is essential also to distinguish between methods in which the values are estimated graphically (from the slope and intercept of the resulting lines) and those in which the values are calculated statistically (least-squares regression). The main objection to the graphical solution is that by this technique the parameter variances are not being estimated (for instance, the utility of the Scatchard plot is a subject of continuing controversy; Connors 1987). To overcome this problem the use of the weighted linear least-squares regression could be applied and, even better, non-linear least-squares regression. Non-linear least-squares regression is based on iteration procedures using specific algorithms.

In conclusion, this study proposes three improved mathematical models for more accurate calculation of binding constants. One of the models described also gives an indication of the extent of complexation. Because linear models are still used extensively in the literature, even though there are proposals that they should be totally replaced by non-linear models, the evaluation of binding constant values by use of non-linear models could be of interest, because these models are free from any assumptions and from practical or theoretical shortcomings.

Appendix

Starting from the cyclodextrin mass balance and the basic equation for the binding constant, transformation gives:

$$CyD = CyD_t - G : CyD = CyD_t - K_{1:1}CyD\ G \Rightarrow$$
$$CyD + K_{1:1}CyD\ G = CyD_t \Rightarrow$$
$$CyD(1 + K_{1:1}G) = CD_t \Rightarrow$$
$$CyD = CyD_t/(1 + K_{1:1}G) = CyD_t/(1 + K_{1:1}[G_t - G : CyD])$$
$$= CyD_t/(1 + K_{1:1}[G_t - CD_t + CD]) \Rightarrow$$

Further transformation gives the equation:

$$K_{1:1}CyD^2 + (K_{1:1}G_t - K_{1:1}C_t + 1)CyD - CyD_t = 0 \quad (A1)$$

The value of CyD can be calculated by solving the quadratic equation ($ax^2 + bx + c = 0; x_{1,2} = [-b \pm \sqrt{(b^2 - 4ac)}]/2a$) :

$$CyD_{1,2} = \{-(K_{1:1}G_t - K_{1:1}CyD_t + 1) \pm \sqrt{[(K_{1:1}G_t - K_{1:1}CyD_t + 1)^2 + 4K_{1:1}CyD_t]}\}/2K_{1:1} \quad (A2)$$

References

Armstrong, D., Nome, F., Spino, L., Golden, T. (1986) Efficient detection and evaluation of cyclodextrin multiple complex formation. J. Am. Chem. Soc. 108: 1418–1423

Connors, K. A. (1987) Binding Constant, The measurement of Molecular Complex Stability. John Wiley, New York, p. 386

Diedrich, F. (1988) Complexation of neutral molecules by cyclophane hosts. Angew. Chem. Int. Ed. Engl. 27: 362–368

Djedaini, F., Perly, B. (1993) Nuclear magnetic resonance of cyclodextrins, derivatives and inclusion compounds. In: Duchene, D. (ed.) New Trends in Cyclodextrins and Derivatives. 1st edn, Edition de Sante, Paris, pp 215–247

Djedaini, F., Lin, S. Z., Perly, B., Wouessidjewe, D. (1990) High-field nuclear magnetic resonance techniques for the investigation of a beta-cyclodextrin:indomethacin inclusion complex. J. Pharm. Sci. 79: 643–646

Ferguson, S. B., Diederich, F. (1986) Electron donor–acceptor interactions in host–guest complexes in organic solutions. Angew. Chem. Int. Ed. Engl. 25: 1127–1128

Loukas, Y. L. (1995) Complexation of haloperidol with a water-soluble β-cyclodextrin polymer: characterization of the inclusion complex in solid state and in aqueous solution. Pharm. Sci. 1: 509–512

Loukas, Y. L. (1997a) Multiple complex formation of fluorescent compounds with cyclodextrins: efficient determination and evaluation of the binding constant with improved fluorimetric studies. J. Phys. Chem. B. 101: 4863–4866

Loukas, Y. L. (1997b) Multiple complex formation of unstable compounds with cyclodextrins: efficient determination and evaluation of the binding constant with improved kinetic studies. Analyst 122: 377–381

Loukas, Y. L., Antoniadou-Vyza, E., Valiraki, A. (1995) Inclusion complexes and stability studies of an organophosphorus insecticide with cyclodextrins: spectrophotometric and kinetic determination of stability constant. Analyst 120: 533–599

Loukas, Y. L., Vraka, V., Gregoriadis, G. (1997) New non-acidic formulations of haloperidol complexed with β-cyclodextrin derivatives. J. Pharm. Biomed. Anal. In press

Miyahara, M., Takahashi, T. (1982) Determination of stability constants of complexes by the non-linear optimization method and analysis of solubility curves. Chem. Pharm. Bull. 30: 288–295

Saenger, W. (1980) Cyclodextrin inclusion complexes in research and industry. Angew. Chem. Int. Ed. Engl. 19: 344–362

Schneider, H. J., Philippi, K., Pohlmann, J. (1984) Structure- and solvent-dependence in the complexation of lipophilic substrates in a water-soluble azacyclophane. Angew. Chem. Int. Ed. Engl. 23: 908–909

Schneider, H. J., Kramer, R., Simova, S., Schneider, U. (1988) Solvent and salt effects on binding constants of organic substrates in macrocyclic host compounds. A general equation measuring hydrophobic binding contributions. J. Am. Chem. Soc. 110: 6442–6448