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Evaluation of the methods for the determination of the stability constant of cyclodextrin–chlorambucil inclusion complexes

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Abstract

The interaction of the antitumor agent chlorambucil (CHL) with three different cyclodextrins (CD), namely methyl-βCD (MeβCD) polymer-βCD (poly-βCD) and γCD, is examined kinetically and spectrophotometrically, monitoring the hydrolysis and the changes in the UV absorbance of CHL respectively, in the presence of increasing concentrations of the examined CD. The stoichiometry coefficient for all the CHL–CD complexes was calculated and found to be 1:1, using the continuous variation method based on the UV data. Also, the stability constant K_{st} for the CHL–CD complexes was calculated and evaluated using the above mentioned two methods, each one based on linear and nonlinear mathematical models. All studies demonstrate that the interaction of CHL with the methylated derivative (MeβCD) is stronger (the highest K_{st} value), probably due to the enhanced hydrophobic character of this derivative. © 1997 Elsevier Science B.V.

Keywords: Chlorambucil; Cyclodextrins; Binding constant; Linear models; Nonlinear models; Continuous variation method

1. Introduction

A well known [1] approach for the chemical stability of sensitive drugs is their complexation with cyclodextrins (CD). CD are α-1,4 linked cyclic oligosaccharides of D-glucopyranose units, known to form non-covalent water-soluble inclusion complexes with a wide variety of drugs, thus improving their solubility and bioavailability. β-Cyclodextrin (βCD) is of appropriate size and shape to interact efficiently with numerous drug substances, but, due to its relatively low aqueous solubility, it exerts various toxic manifestations when this compound is administered parenterally. CD derivatives, such as methylated, hydroxypropylated or polymers, are used extensively due to their higher aqueous solubility compared to natural CD, resulting in lower haemolytic activity and irritation [2].

Because of the increased interest and their inherent usefulness, different studies have been done to evaluate the complexation procedure, the stability constant and the stoichiometries of the complexes formed. Most of these studies assume a 1:1 molar ratio between the CD and the guest

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molecule (G) of interest. A distinct problem, apparent from the literature, is the deviations observed for the calculated values of the stability constants and the stoiciometries which derive by using different methods for the same G–CD system. For example, the K_{st} of the *p*-nitrophenol complex with αCD is 126M^{-1} when calculated by titration calorimetry and 250 M^{-1} when calculated by spectrophotometry [3]. Moreover, the 1:1 inclusion complex of *p*-nitrophenolate with αCD has a K_{st} value of 1590 M^{-1} when calculated by optical rotation [4] and a value of 3550 M^{-1} when calculated by gel filtration [5]. Also, it is becoming evident that assuming 1:1, 1:2 or 2:1 complexes without having some experimental evidence, can lead to erroneous results. For example, in the literature one group [6] assumes 1:1 complexation of prostaglandin B_1 with αCD and another group [7] 1:2, with significant deviation in their results. Obviously, this can significantly alter the interpretation of the conclusions in studies involving complexation with CD. Furthermore, deviations appear in studies for the definition of the complex molecular structure, i.e. which part of the G molecule is included in the CD cavity; for instance, the indomethacin molecule appears to enter the cavity of βCD with the *p*-chlorobenzyl moiety, according to one group [8], or with the six-member ring of the indole unit according to another [9].

A number of different physicochemical methods are described in the literature for determination of the binding constant based on techniques such as ^{1}H NMR, conductometric titrations, potentiometric, spectrophotometric and fluorometric methods, solubility and competitive indicator binding. For the determination of the binding constant in 1:1 complexes, a number of linear procedures have been used, but most of these suffer from theoretical and practical drawbacks [10], such as assumed concentrations of the interacting moieties and products, poor solubility of certain compounds, a boundary condition (saturation binding) with respect to the ratio of the concentrations of the two binding partners and the occasional formation of dimers. On the other hand, Diederich [11] suggests that nonlinear procedures are free of the above assumptions and have much broader applicability, so that such procedures are likely to displace the evaluations carried out according to the Benesi-Hildebrand and to the Scott or Scatchard linear models.

In the present study it is demonstrated that kinetic and spectrophotometric studies of chlorambucil (CHL) in the presence of CD can be used to determine and evaluate the binding constants for the CHL–CD systems, using both linear and nonlinear mathematical models. Also, the complex stoichiometry, in all cases, is calculated using the continuous variation method based on the UV data.

2. Experimental

2.1. Materials and instrumentation

CHL was obtained from Sigma (Poole, Dorset, UK); γCD, MeβCD and poly-βCD (MW 4000–4500, crosslinked with 1-chloro-2,3-epoxy propane) were from Cyclolab (Budapest, Hungary); MeβCd has a degree of substitution (DS) of 1.8 (number of methyl groups per unit of anhydroglucose) and a relative molecular mass (M_r) of 1325. The DS value (a measure of the extent to which the reactive hydroxyls in each glucose unit of the ring have been submitted) obtained by digital integration, was confirmed from the ^{1}H NMR spectrum of MeβCD in deuterium oxide. Double distilled water was obtained through a MilliQ system, (Waters). All other reagents were of analytical grade. Studies on the hydrolysis kinetics and the spectrophotometric behavior of CHL in aqueous buffered solutions in the presence of CD were performed and monitored in a Compuspec UV/visible spectrophotometer (Wallac) connected to a personal computer.

2.2. Kinetic and spectrophotometric studies

A stock solution of CHL in methanol-water (1:1, v/v) was prepared and a standard quantity of it was added to acetate buffer solutions (pH = 4.15) containing increasing concentrations of the CD examined. At appropriate intervals, samples were taken and analyzed for remaining CHL by

monitoring spectrophotometrically the decrease in absorbance at 255.5 nm.

Also, from the stock solution of CHL a standard aliquot was pipetted into flasks, containing increasing concentrations of buffered solutions of the examined CD. The content of each flask was diluted, mixed quickly and measured spectrophotometrically at room temperature (ca. 22°C), against a reference containing the CD in the same concentration, in order that both solutions should have the same refractive index.

2.3. Continuous variation plot

Before proceeding with the calculation of the binding constants, it is important that the stoichiometry of the complexes is calculated. A reliable determination of the complex stoichiometry is provided by the continuous variation technique (Job plot) [12], based on the difference in absorbance $\Delta A(\Delta A = A_0 - A)$ of CHL observed in the presence and absence of CD. Equimolar solutions of the G (CHL in the present work) and of each of the corresponding CD were prepared and mixed to a standard volume and proportions to ensure that the total concentration remained constant ($[G]_t = [CD]_t = M$).

ΔA values in the CHL preparations were calculated by measuring the absorbance of CHL in the absence (A_0) and presence (A) of the corresponding concentration of CD. Also an equimolar aqueous solution of each CD was used as blank, to take into account its refractive index. Subsequently, $\Delta A[CHL]_t$ was plotted for the corresponding CD against r; $r = \dfrac{[G]_t}{[G]_t + [CD]_t}$ where G denotes CHL; the continuous variation method is described in detail elsewhere [13].

3. Results and discussion

3.1. Kinetic determination of the stability constants

The resulting continuous variation plots (Fig. 1) demonstrate that since r has a maximum value of almost 0.5 for all the CHL–CD complexes,

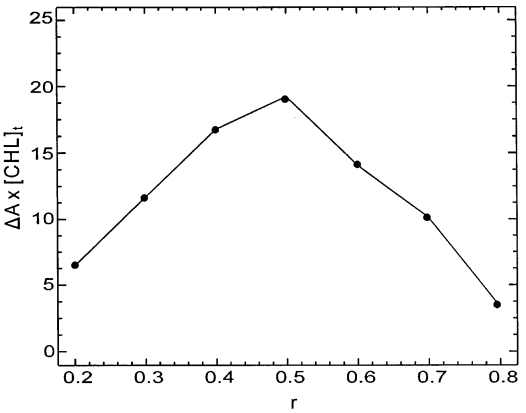
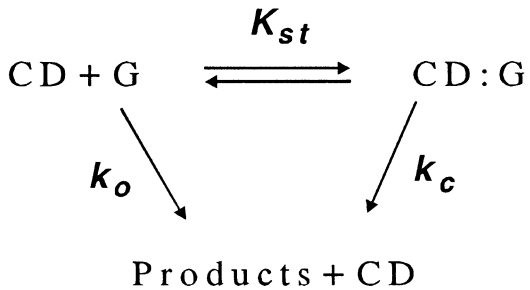


Fig. 1. Continuous variation plot (Job plot) of CHL–MeβCD. (the other two complexes (not shown) present almost the same behaviour).

these complexes have a 1:1 stoichiometry. The reaction mechanism, involving the formation of a 1:1 (molar ratio) inclusion complex, is illustrated in Scheme 1, where CD is the cyclodextrin molecule (γCD, MeβCD or Poly-βCD), G is CHL in the present work, k_o is the degradation rate constant for the non-catalyzed reaction (i.e. in the absence of CD) and k_c is the degredation rate constant of the guest in the form of the inclusion complex. Linear models which describe the kinetic behaviour of guests in the presence of CD are usually solved according to Lineweaver-Burk [14] or Eadie [15] equations.

The observed reaction rate for CHL degradation in the presence of CD is a weighted average of the rate of reaction of free CHL and the rate of reaction of CHL included in the CD, therefore K_{st}



Scheme 1.

Table 1
Stability constants values for the CHL–CD systems at 37°C, based on the kinetic and UV methods

Complex	Kinetic model (Eq. (2)) M^{-1}	Kinetic model (Eq. (3)) M^{-1}	UV model (Eq. (5)) M^{-1}	UV model (Eq. (11)) M^{-1}
CHL–MeβCD	7540 ($\pm$350)	9370 ($\pm$480)	8715 ($\pm$396)	9150 ($\pm$425)
CHL–poly-βCD	5955 ($\pm$290)	6820 (310)	6370 ($\pm$295)	6115 ($\pm$322)
CHL–γCD	2500 ($\pm$195)	3320 ($\pm$220)	3605 ($\pm$256)	2890 ($\pm$205)

can be determined from the dependence of the observed rate constant on the concentration of added CD. The actual measurable rate constant is:

$$k_{obs} = k_o + \frac{(k_c - k_o)[CD]}{1/K_{st} + [CD]} \quad (1)$$

A graphical solution of Eq. (1) corresponds to one half of one branch of a typical rectangular hyperbola, which can be easily transformed to linear form by the Lineweaver-Burk double reciprocal transformation, as shown in Eq. (2):

$$\frac{1}{k_o - k_{obs}} = \frac{1}{K_{st}(k_o - k_c)}\frac{1}{[CD]} + \frac{1}{k_o - k_c} \quad (2)$$

A graphical solution of $1/(k_o - k_{0bs})$ versus $1/[CD]$ gives a line (not shown) with $1/K_{st}(k_o - k_{obs})$ as the slope and $1/(k_o - k_c)$ as the intercept from which K_{st} is derived (Table 1).

In addition to the known linear models, a new nonlinear model (Eq. (3)) has been described recently [16], avoiding the theoretical and practical drawbacks of the linear models, viz.:

$$\Delta k_{obs} = K_{st}\left(1 - \frac{\Delta k_{obs}}{\Delta k_c}\right)\left\{[CD]_t - \frac{[G]_t \Delta k_{obs}}{\Delta k_c}\right\}\Delta k_c \quad (3)$$

where $\Delta k_{obs} = (k_o - k_{obs})$ and $\Delta k_c = (k_o - k_c)$.

Eq. (3) involves no approximation of the concentrations of the two compounds (CD and G) and correlates the initial total concentrations $[G]_t$ and $[CD]_t$ with the rate constants k_o and k_{obs}. The unknown parameters K_{st} and k_c can then be calculated according to this model.

3.2. Spectrophotometric determination of the stability constants

The stability constant of CHL–CD complexes were also studied in aqueous buffered solution by UV spectroscopy. The zero order UV-absorption spectra of CHL exhibit a bathochromic shift in λ_{max} in the presence of CD (Fig. 2). This is probably due to the high electron density inside the hydrophobic CD cavity, which creates a partial shielding of the excitable electrons of CHL, when CHL is included inside the CD cavity. The change in absorbance (ΔA) at 255.5 nm as a function of the CD concentration examined agrees with the typical binding isotherm (Eq. (4)):

$$\frac{\Delta A}{b} = \frac{[CHL]K_{st}\Delta\varepsilon_{11}[CD]}{1 + K_{st}[CHL]} \quad (4)$$

Transformation of Eq. (4) based on the Benesi-Hildebrand [17] model gives:

$$\frac{b}{\Delta A} = \frac{1}{[CHL]K_{st}\Delta\varepsilon_{11}[CD]} + \frac{1}{[CHL]\Delta\varepsilon_{11}} \quad (5)$$

where b is the path length ($b = 1$ cm) and $\Delta\varepsilon_{11} = (\varepsilon_{11} - \varepsilon_{CHL} - \varepsilon_{CD})$. The correlation of ΔA^{-1} versus $[CD]^{-1}$ gives a straight line (not shown) with

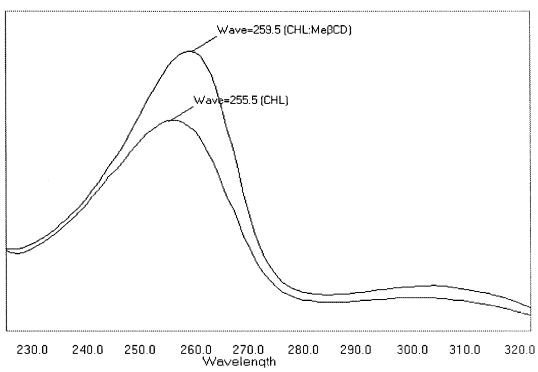


Fig. 2. Bathochromic shift of the CHL–MeβCD λ_{max} compared to the λ_{max} of the free CHL.

$1/[CHL]K_{st}\Delta\epsilon_{11}$ as the slope and $1/[CHL]\Delta\varepsilon_{11}$ as the intercept, from which K_{st} is derived (Table 1).

In addition to the commonly used linear model (Benesi-Hildebrand), a nonlinear model can be derived (Y.L. Loukas, Unpublished results) starting from the basic equations of mass balance for both compounds (G and CD), the stability constant equation, and the Lambert-Beer law for each substance:

$$[G] = [G]_t - [CD:G] \quad (6)$$

$$[CD] = [CD]_t - [CD:G] \quad (7)$$

$$K_{st} = \frac{[CD:G]}{[CD][G]} \quad (8)$$

$$A_o = \varepsilon_G b[G] + \varepsilon_{CD} b[CD] + \varepsilon_{CD:G} b[CD:G] \quad (9)$$

Substitution of the mass balance from Eq. (6) and Eqs. (7)–(9) and setting the CD absorbance equal to zero (since CD do not absorb), the measured absorbance then becomes:

$$A = \varepsilon_G b G_t + \Delta\varepsilon_{11} b[CD:G] \quad (10)$$

Combining the above equations and after consecutive transformations, Eq. (11) is derived:

$$\Delta A = \frac{[G]_t K_{st}\Delta\varepsilon_{11}[CD]_t - [G]_t K_{st}[\Delta A]}{1 + K_{st}[CD]_t + K_{st}\dfrac{\Delta A}{\Delta\varepsilon_{11}}} \quad (11)$$

Eq. (11) is the final nonlinear model which correlates the difference in absorbance with the initial total concentration of G (G_t) and of CD (CD_t) (Fig. 3). The unknown parameters K_{st} and $\Delta\varepsilon_{11}$ can be calculated by the model according to the nonlinear least-squares regression as above.

From the values in Table 1 the following conclusions can be drawn: (a) judging from the R^2 values ($R^2 > 0.87$ in all cases), the models appear to fit the observed values reasonably well. (b) Different methods (kinetic and spectrophotometric) and different mathematical models (linear and nonlinear) give different (albeit modest) K_{st} values. The variability is higher between methods (kinetic or UV) than between models (linear or nonlinear). However, in all cases the order of the numerical values is constant (MeβCD > poly-βCD > γCD). (c) The order in the K_{st} values suggests that the driving force for complexation is the hydrophobic

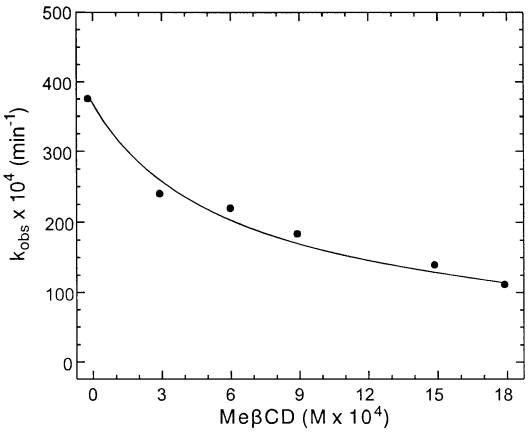


Fig. 3. Plot of k_{obs} vs. MeβCd concentration, illustrating the nonlinear behavior of Eq. (3). (the other two complexes (not shown) present almost the same graphical solution).

character, rather than the formation of additional H bonds. This can be supported by the fact that the methylated derivative has an increased hydrophobic character and fewer OH groups, compared to poly-βCD, due to the selective replacement of the internal OH by the methyl groups. Furthermore, the hydrophobic–hydrophobic interaction inside the γCD cavity is also reduced due to its bigger size, which probably increases the degrees of freedom (transational and rotational) of the CHL molecule. It is evident that, for comparative studies of G–CD complexes (the same G with various CD), a physicochemical parameter (corresponding to the CD or the G) with a more distinct change, must be chosen. When this parameter has been found, the same method and the same mathematical model should be used for the calculated values to be comparable and to lead to correct conclusions, for the characterization of the complex and the complexation procedure.

4. The method of choice

Connors [18] concludes that if the calculated stoichiometry of a complex is wrong, then the calculated stability constant is wrong too and when there are (statistically) significantly different

results from two independent methods, then the assumed stoichiometry is wrong. From the present and other studies, the following conclusions can be drawn: (a) the most important factor is the calculation of the complex stoichiometry, which in many cases is simplified to 1:1, even though there are higher order complexes, leading to erroneous results. (b) Deviations of the calculated values could be eliminated by using the same experimental procedure for all the examined G–CD complexes. (c) The variability of the binding constant values should be examined in order to evaluate the reproducibility and repeatability of the method. (d) Finally, the known linear models, could be replaced [11] by nonlinear ones, because the former are based on assumptions (for instance, the total CD concentration is equal to the free CD concentration when $[CD]_t \gg [G]_t$) leading to theoretical and practical shortcomings.

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