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Cyclodextrin Binding Constants as a Function of pH for Compounds with Multiple pK_a values

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Abstract

Complex formation between cyclodextrins and ionizable guest molecules depends on pH. The neutral species of an ionizable guest molecule in general has the highest affinity for the cyclodextrin cavity, but ionized species will also be able to form complexes with cyclodextrins. This work presents a theoretical expression for the relationship between the stability constant and pH for interaction between neutral cyclodextrins and ionizable guest molecules with multiple pK_a values. Input parameters for the theoretical expression are pK_a values of the guest molecule and stability constants for the complex at specific pH values. The pH profile of the stability constant for a complex depends on the acid-base properties of the guest and the closeness of the pK_a values, and examples of pH profiles for polyprotic acids, bases and amphoteric guests are shown. Empirical data sets from the literature were used to confirm the accuracy of the theoretical expression, and Monte Carlo simulations were used to validate that the theoretical expression yield a good fit to empirical data. Lastly, an experimental protocol was suggested, and a freely available graphical user interface is presented to facilitate easy use of the theoretical expression.

Keywords: Distribution of species; Ionization; Hydrophobic interactions; Monte Carlo simulations; Drug Development; Graphical User Interface;

1. Introduction

Cyclodextrins (CDs) are well-known for their ability to form inclusion complexes (Loftsson and Brewster, 2012). Natural CDs consist of (α-1,4)-linked glucopyranose units forming a cone-like structure with a hydrophilic outside and a more hydrophobic cavity (Loftsson and Brewster, 2010). Hydrophobic guest molecules will through non-covalent interactions be attracted to the hydrophobic cavity of the CD (Jansook et al., 2018), and industries like the pharmaceutical, food and cosmetic, use CDs as excipients in their products (Astray et al., 2009; Kurkov et al., 2011).

One main advantage of CDs are their ability to improve the apparent physicochemical properties, e.g. solubility and stability, of the guest molecules without altering the original properties of the guest molecule (Loftsson and Brewster, 2010, 1996). This is extremely useful in the pharmaceutical industry, although it is not limited to this particular industry. The physicochemical properties of molecules are important in early stages of drug and formulation development (Bhattachar et al., 2006; Schönherr et al., 2015). Many potential drug molecules are hydrophobic compounds with low solubility, which exhibit poor bioavailability (Williams et al., 2013). Complex formation with CDs improves the apparent solubility without changing the hydrophobic nature of the potential drug, which ensures the ability of the drug to cross lipophilic barriers in the body.

Complex formation between CD and guest molecules is an equilibrium reaction. The stability constant for CD complexes are used to evaluate the complexation, and it is an important characteristic (Loftsson and Brewster, 2010). For example, during drug formulation an extended knowledge of the complexation is necessary to define an optimal robust formulation under all conditions of use.

However, in practice complex formation between CD and guest is often not the only equilibrium that must be considered. The acid-base equilibria is an important additional consideration because

many guest molecules are ionizable molecules, and their charge depends on the pH of the solution (Babic et al., 2007). There might be one or more relevant acid-base equilibria to consider when working with CD complexation. The stability constants for CD complexes with ionizable guest molecules depend on pH (Al Omari et al., 2008; Kuwabara et al., 2006; Samuelsen et al., 2019; Veiga et al., 1996; Zheng et al., 2013). The neutral and ionized version of the same molecule will have different affinities for the CD. The neutral species will in general have a higher affinity for the CD cavity, as it will be more hydrophobic, compared to the ionized form of the same molecule (Cirri et al., 2006). The difference in affinity is difficult to predict, but it depends on the hydrophilic character of the guest and the position of the ionizable group relative to where the complexation occurs (Astray et al., 2009).

The stability constant will change as the experimental conditions change, e.g. the pH of the solution changes. During drug formulation, where the stability of a formulation must be guaranteed over a pH and temperature range, it is important to understand how the apparent stability will change. Also in the food industry, the effect of pH is important (López-Nicolás et al., 2009). It is possible to establish the effect of pH by conducting experiments to determine the stability constant in the entire range of interest, but this requires excessive experimentation, usage of compound and is time consuming (Ghasemi et al., 2011). Thus, one alternative is a combination of experimental determination and equilibrium theories to predict the stability constant for different pH ranges.

Understanding the interplay between the complexation equilibrium and acid-base equilibria can be used to optimize work with CDs. In this work, theoretical expressions for the relation between the stability constant and pH will be formalized. This work will serve as a framework for necessary considerations when working with CD equilibrium systems influenced by pH. Based on the theoretical expression, a graphical user interface was developed to facilitate easy use of the theoretical work.

2. Equilibria situations

Complex formation between CD and guest molecules (D) is an equilibrium reaction, and it can be written as a simple equilibrium diagram (Eq. 1).

$$CD + D \rightleftharpoons CD{:}D \quad (1)$$

The equilibrium reaction can be described quantitatively in terms of the stability constant for the complex.

$$K = \frac{[CD{:}Guest]}{[CD][Guest]} \quad (2)$$

The stability constant is used as an important characteristic during drug formulation, and thus it is essential to understand this value.

In solution, it is possible to have several equilibria simultaneously leading to complex equilibria diagrams. Many guest molecules are ionizable compounds, and the pH of the solution determines the distribution between the various species of the guest molecule, i.e. the guest molecule exists in different ionization states (e.g H^+D^- and D^-). In a simple example, the guest molecule has one pK_a value, and thus two ionization states (Eq. 3).

$$HD \rightleftharpoons H^+ + D^- \quad (3)$$

Both the ionized (D^-) and neutral version (HD) of the guest can interact with the CD, though they will have different affinity for the CD cavity. It is possible to write up one equilibrium diagram, which explains the relation between all the different equilibrium reactions in the solution (Scheme 1). The equilibrium diagram shows that the acid base equilibria are linked to the CD complex equilibria.

Guest molecules can also have more than one pK_a value increasing the number of equilibria in the solution. Guest molecules that are either polyprotic acids, bases or amphoteric compounds may have more than two ionization states. Zwitterions are a special case, since they have internal charges, but a net charge of zero. In the relations to the theories presented in this work, it is fair to consider zwitterions as amphoteric compounds with one neutral state. Given that the differences between pK_a values are greater than four, stepwise dissociation can be considered (Eq. 4 and 5).

$$H_2D^+ \rightleftharpoons H^+ + HD \quad (4)$$

$$HD \rightleftharpoons H^+ + D^- \quad (5)$$

When two or more pK_a values are very close to each other, it is possible to have a more complex mixture of ionization states for the guest molecule (Fig. 1B).

The increasing number of equilibria will lead to a more complex equilibrium diagram when also considering the interaction with CDs (Scheme 2). An increasing number of pK_a values will lead to a further complication of the equilibrium diagram. In practice, when the pK_a values are far apart, it is possible to only consider two ionization states of the guest, as only two ionization states will be present simultaneously at a given pH value (Fig. 1A), whereas close pK_a values will show a more complex mixture of ionization states, where multiple species will be present simultaneously (Fig. 1B).

The distribution of species as a function of pH depend solely on the pK_a values of the guest, and it will look similar for polyprotic acids, bases and amphoteric molecules. The charges of the various species must be determined by looking at the structure of the molecule. The neutral species of guest molecule will generally have the strongest interaction with the CD, as it will be more hydrophobic (Cirri et al., 2006).

As demonstrated in Fig. 1, the closeness of the pK_a values determines the complexity of the distribution of species. Increasing the number of pK_a values increases the complexity, and even more so when the pK_a values are close to each other (Fig. 2). Depending on the pK_a values, it is possible to have pH values where multiple species of the molecule exist simultaneously. For cases where the pK_a values are close, multiple species co-exist, and thus several pK_a values will influence the stability constant simultaneously.

In a solution, many equilibria can be present simultaneously, and they will all affect the apparent stability constant. When the guest molecule is ionizable, the apparent stability constant will be a function of pH. This can be exploited to manipulate the apparent stability constant of the solution by changing the pH of the solution. Based on the equilibrium diagrams, it is possible to write theoretical expressions for the relation between the different stability constants and the pH of the solution. This insight can help formulation scientists define the most optimal and robust range for a formulation using CDs as a solubilizing excipient.

3. Theoretical relation between stability constant and equilibrium terms

3.1 Theoretical expression for stability constant as a function of pH

Previously, a theoretical expression for the stability constant, K_B, for the complexation between CD:guest as a function of pH was derived and presented (Samuelsen et al., 2019). The expression shows the correlation between pH, pK_a and the stability constants for the complexation between CD and the charged and neutral species of guest molecule, K_{ion} and K_{neu}. However, the previous expression is limited to guest molecules with only one pK_a value. Potentially, the guest has multiple pK_a values, and all of the species will be able to form complexes to some extent. It is possible to write up an equilibrium diagram of how these species are linked.

The overall equilibrium for a reaction between CD and drug, D, can be written, and the stability constant, K_B, can be expressed in terms of the concentration of the reacting species.

$$CD + D \rightleftharpoons CD{:}D \tag{6}$$

$$K_B = \frac{[CD{:}D]}{[D][CD]} \tag{7}$$

To exemplify, let us consider an amphoteric compound. An amphoteric compound consists of at least three species, and for each species, we can write up an equilibrium reaction for the complex formation with CD.

$$CD + H_2D^+ \rightleftharpoons CD{:}H_2D^+ \tag{8}$$

$$CD + HD \rightleftharpoons CD{:}HD \tag{9}$$

$$CD + D^- \rightleftharpoons CD{:}D^- \tag{10}$$

Each of the equilibrium has a corresponding stability constant.

$$K_1 = \frac{[CD{:}H_2D^+]}{[CD][H_2D^+]} \tag{11}$$

$$K_2 = \frac{[CD{:}HD]}{[CD][HD]} \tag{12}$$

$$K_3 = \frac{[CD{:}D^-]}{[CD][D^-]} \tag{13}$$

The overall stability constant, K_B, can be expressed in terms of the individual equilibria.

$$K_B = \frac{[CD{:}D]}{[CD][D]} = \frac{[CD{:}H_2D^+]+[CD{:}HD]+[CD{:}D^-]}{[CD]([H_2D^+]+[HD]+[D^-])} \tag{14}$$

This expression can be rearranged.

$$= \frac{[CD{:}H_2D^+]}{[CD]([H_2D^+]+[HD]+[D^-])} + \frac{[CD{:}HD]}{[CD]([H_2D^+]+[HD]+[D^-])} + \frac{[CD{:}D^-]}{[CD]([H_2D^+]+[HD]+[D^-])}$$

$$= \frac{[CD{:}H_2D^+]}{[CD][[H_2D^+]} \frac{[H_2D^+]}{[H_2D^+]+[HD]+[D^-]} + \frac{[CD{:}HD]}{[CD][HD]} \frac{[HD]}{[H_2D^+]+[HD]+[D^-]} + \frac{[CD{:}D^-]}{[CD][D^-]} \frac{[D^-]}{[H_2D^+]+[HD]+[D^-]} \quad (15)$$

Parts of this equation can now be substituted with the equilibrium constants in Eq. 11-13.

$$K_B = K_1 \frac{[H_2D^+]}{[H_2D^+]+[HD]+[D^-]} + K_2 \frac{[HD]}{[H_2D^+]+[HD]+[D^-]} + K_3 \frac{[D^-]}{[H_2D^+]+[HD]+[D^-]} \quad (16)$$

The fractions in equation 16 represents the dissociation degree of the drug species, α. The total concentration of the drug, C_D, is equal to the sum of the concentrations of the three species.

$$C_D = [H_2D^+] + [HD] + [D^-] \quad (17)$$

$$\alpha_1 = \frac{[H_2D^+]}{C_D} \quad (18)$$

Similar, expression for α_2 and α_3 can be written. Thus, the expression in Eq. 16 can be further simplified.

$$K_B = K_1\alpha_1 + K_2\alpha_2 + K_3\alpha_3 \quad (19)$$

This overall equilibrium constant, K_B, is now expressed in terms of the equilibrium constant for each species and the dissociation degree. The dissociation degree is expressed in terms of the concentration of the species as given in Eq. 18. This can be rearranged.

$$1/\alpha_1 = \frac{C_D}{[H_2D^+]} = \frac{[H_2D^+]+[HD]+[D^-]}{[H_2D^+]} = \frac{[H_2D^+]}{[H_2D^+]} + \frac{[HD]}{[H_2D^+]} + \frac{[D^-]}{[H_2D^+]} \quad (20)$$

The dissociation degree of the drug is linked to the drug equilibrium (Eq. 21).

$$K_{a1} = \frac{[H^+][HD]}{[H_2D^+]} = [H^+]\frac{[HD]}{[H_2D^+]} \quad (21)$$

By isolating the concentration of the drug species ($[HD]/[H_2D^+]$) and using the definitions for pK_a and pH, the dissociation degree can be expressed in terms of pH and pK_a.

$$^1/_{\alpha_1} = 1 + 10^{(pH-pK_{a1})} + \frac{[D^-]}{[H_2D^+]} \quad (22)$$

The last fraction in Eq. 22 can also be expressed in terms of pH and pK_a. First, we consider the equilibrium between the two species H_2D^+ and D^-.

$$H_2D^+ \rightleftharpoons 2H^+ + D^- \quad (23)$$

The equilibrium has a corresponding equilibrium constant.

$$K_{a1}K_{a2} = \frac{[H^+]^2[D^-]}{[H_2D^+]} = [H^+]^2 \frac{[D^-]}{[H_2D^+]} \quad (24)$$

Again, it is possible to isolate the concentration of the species and use the definitions of pH and pK_a.

$$2pH = (pK_{a1} + pK_{a2}) + log\frac{[D^-]}{[H_2D^+]} \quad (25)$$

$$\frac{[D^-]}{[H_2D^+]} = 10^{(2pH-(pK_{a1}+pK_{a2}))} \quad (26)$$

Now, it is possible to express the dissociation degree in terms of pH and pK_a values.

$$^1/_{\alpha_1} = 1 + 10^{(pH-pK_{a1})} + 10^{(2pH-(pK_{a1}+pK_{a2}))} \quad (27)$$

Similar expression can be written for α_2 and α_3.

$$^1/_{\alpha_2} = \frac{[H_2D^+]}{[HD]} + \frac{[HD]}{[HD]} + \frac{[D^-]}{[HD]} = 10^{(pK_{a1}-pH)} + 1 + 10^{(pH-pK_{a2})} \quad (28)$$

$$^1/_{\alpha_3} = \frac{[H_2D^+]}{[D^-]} + \frac{[HD]}{[D^-]} + \frac{[D^-]}{[D^-]} = 10^{((pK_{a1}+pK_{a2})-2pH)} + 10^{pK_{a2}-pH} + 1 \quad (29)$$

By combining Eq. 19, 27, 28 and 29, it is possible to express K_B as a function of pH based on the stability constants and the pK_a values.

For compounds with more than two pK_a values, a similar approach can be used to further complicate the equilibrium diagram (Scheme 3), which complicates the expression in Eq. 19.

For each additional pK_a value, a new term $K_x\alpha_x$ must be added to the equation for the overall stability constant.

$$K_B = K_1\alpha_1 + K_2\alpha_2 + K_3\alpha_3 + K_x\alpha_x + \cdots \quad (30)$$

The total concentration of drug, C_D, also changes based on the number of pK_a values. The number of species is equal to the number of pK_a values plus one, and the total concentration of the drug can be written.

$$C_D = \left[H_y D^{(y-1)+}\right] + \cdots \quad (31)$$

Where y is the number of pK_a values of the drug, and the term must be written for all species. Similar, equivalent number of expressions can be written for the dissociation degree $\alpha_{1\text{-}4}$.

3.1.1 Examples of the overall stability constant as a function of pH for acids, bases and amphoteric compounds

The distribution of species as a function of pH will look similar for polyprotic acids and bases and amphoteric compounds, but this is not the case for the overall stability constant as a function of pH. All species of the guest molecule is expected to interact with the CD in a solution, though the stability constant must be expected to be highest for the interaction between CD and neutral species. Thus, it follows the acid-base properties of the guest is important for the pH profile of the overall stability constant. To exemplify this, the overall stability constant as a function of pH was calculated considering three cases; 1) acidic guest (Fig. 3AB), 2) basic guest (Fig. 3CD), and 3) amphoteric guest (Fig. 3EF). For the three cases, two different set of pK_a values will be used; 3.0,

7.0 and 11.0 or 4.0, 5.0 and 6.0. The stability constants were chosen to illustrate difference between the acid-base properties and the closeness of pK_a values. The further apart the pK_a values are the more defined plateaus can be identified for the overall stability constant (Fig. 3ACE), whereas closeness of the pK_a values resulted in fewer plateaus. The example demonstrates that the pH profile for the overall stability constant can be quite complex. It depends on acid-base properties of the guest, the number and closeness of the pK_a values and the stability constant for each of the species.

With the information provided in Fig. 3, a formulation scientist working with a CD based formulation would have a strong tool as a support to identify the pH range that would lead to the most robust formulation. For a formulation scientist the purpose of using CDs is most often to increase the apparent solubility of a formulation as well as ensuring high stability. The optimal pH range must guarantee a robust stability constant, i.e. one the does not change much with small changes in pH, and a high solubility, which depends on the pK_a value of the guest. As an example, for acidic compounds like the ones presented in Fig. 3A, the formulation will be robust in the ranges pH >2, 4-6, 8-10 and <12. The solubility of the guest will increase with the ionization. The optimal pH range with respect to solubility will be a trade off between having a high stability constant and an ionized species. For the compounds presented in Fig. 3E and 3F, it is clear that closeness of the pK_a values matters for the optimal range, so for a compound in Fig. 3E, it is easier to define a robust range than for a compound like the one in Fig. 3F.

3.1.2 Cases from the literature

In previous work, a theoretical expression for the overall stability constant as a function of pH for CD complexes was derived for guest molecules with only one pK_a value (Samuelsen et al., 2019). A

good agreement was seen between empirical data and the theoretical expression. Guest molecules with more than one pK_a values is not uncommon, though few studies have reported the stability constant at several pH values for this type of guest molecules. Thus, it is difficult to obtain relevant data set to confirm the theoretical expression.

Sildenafil forms inclusion complexes with β-CD (Al Omari et al., 2006). Sildenafil is an amphoteric compound which has two pharmaceutical relevant pK_a values of 7.10 and 9.84 (Al Omari et al., 2006). Sildenafil is considered neutral around pH 8.5, but charged species will exist simultaneously (Fig. 4). The apparent stability constant is determined at five different pH values, and as expected the neutral form of the molecule has the largest stability constant. Based on the data points, it is possible to find K_1, K_2 and K_3, and thus plot the overall stability constant as a function of pH, and compare this theoretical expressions to the data points (Fig. 5). $K_{1\text{-}3}$ are the stability constants for the binding between CD and each of the individual species, and they are found by identifying the pH range where the molecule predominantly exists as one species. K_1 must be determined in the pH range 0-5, K_2 at approximately pH 8.5 and K_3 in the range pH 12-14. There is a good agreement between data points and the theoretical expression.

Instead of comparing the theoretical expression to the empirical data, which requires empirical stability constants at specific pH values, another approach is to fit the data to the theoretical expression and estimate $K_{1\text{-}3}$. A Monte Carlo analysis was performed to estimate the uncertainties on the three parameters $K_{1\text{-}3}$. Based on the empirical data from Al Omari et al (2006), 1000 simulated data sets were generated assuming normal distribution. The 1000 simulated data sets were fitted to the theoretical expression to obtain best-fit parameters for $K_{1\text{-}3}$ and their standard deviation. The results showed a large uncertainty in estimation of especially K_1 and K_3 (Fig. 6). The estimated parameters were 30±19 M^{-1}, 159±17 M^{-1} and 41±23 M^{-1}. Both variance in data and between data

and model were considered, and this variance describes the behavior of all data points. Uncertainty in the fit must be expected when three parameters are estimated based on only five data points.

The interaction between fisetin and β-CD has been studied, and the stability constant depends on pH (Guzzo et al., 2006). Fisetin is a polyprotic acid. The pK_a values of fisetin are 8.87, 10.31 and 13.20 (Guzzo et al., 2006), and the interaction between CD and fisetin are expected to be highest at acidic pH, where the guest will be neutral (Fig. 7). Due to the closeness of the pK_a values, three species exist simultaneously. The measured stability constants are 900 M^{-1} (pH 4.0), 860 M^{-1} (pH 6.5) and 240 M^{-1} (pH 12.0) (Guzzo et al., 2006). Optimally, the $K_{1\text{-}4}$ values should be found at pH 0-6, 9.5, 11.8 and 14. Data are not available for pH 9.5 and 14. By considering fisetin only in the pH range 0-12, it is possible to consider fisetin as a polyprotic acid with two pK_a values. However, the stability constant for the interaction between Fis^- and CD is still unknown. This is a challenge for plotting the overall stability constant as a function of pH in the range 0-12. However, it is expected that the stability constant for CD:Fis^- will be within the range 240-900 M^{-1} due to its ionization state. By guessing at the stability constant around pH 9.5, it is possible to plot the theoretical expression as a function of pH (Fig. 8), though it is important to highlight that this is at best an approximation. Due to the closeness of the pK_a values, and the fact that fisetin is a polyprotic acid, the plot yielded no clearly defined plateau for the stability constant K_2. It was not possible to estimate the three parameters by fitting the theoretical expression to data, as only three data points exist.

3.3 Protocol for experiments needed and presentation of GUI

Experimental determination of the stability constant ensures exact knowledge of the stability constant for specific experimental conditions. However, experiments can be expensive and time

consuming, and thus alternatives such as combination of experiments and theoretical models may improve the efficiency. The alternative must be cheaper, faster and easy to use in order to have an impact. Based on the theoretical models presented in this work, we have developed a graphical user interface, which can help users estimate the overall stability constant for a CD:guest interaction as a function of pH, and thereby support identification of robust pH ranges for a given CD based formulation.

In order to estimate the overall stability constant as a function of pH, several inputs, based on empirical data, are needed. The inputs needed are the pK_a values of the guest molecule and the stability constants for each of the interaction between the various species of guest and CD. Often, the pK_a values are estimated or determined during early drug discovery, and these values are known prior to CDs being considered as a formulation strategy. The pK_a values can be determined by potentiometry, spectrophotometry, solubility, NMR, liquid chromatography, electrophoresis or calorimetry (Reijenga et al., 2013). When initiating a formulation strategy with CDs, the stability constant must be measured. The stability constant for various species of guest and CD can be determined by phase-solubility, NMR or calorimetry among others (Loftsson and Brewster, 1996). The stability constant should be measured in conditions closely matching the final formulation conditions, e.g. same buffer and tonicity adjuster, if possible. Simulations showed that careful considerations should be made when deciding the number of data points and at what pH values to determine the data points. The optimal solution is to determine the data points as close to the pH, where only one species exist, as possible, and ensuring a low variance in each data point. The alternative is having a larger number of data points (above 14) evenly distributed in the entire pH range, but ensuring low variance of the data points around the non-linear part of the curve. By determining the stability constant at suitable pH values, it will be possible to use the theoretical

expression presented in this work. If this is done early in the formulation process, it can be used as a valuable tool to further optimize the drug formulation.

To exemplify, the fisetin:CD complex is used as an example. In order to get the overall stability constant as a function of pH for fisetin:CD, the three pK_a values must be determined. The pK_a values are 8.87, 10.31 and 13.20, and four different species of the molecule will exist depending on the pH, and thus four stability constants must be determined. Based on the pK_a values, it is possible to plot the distribution of species as a function of pH. This is a helpful tool to identify at which pH values the stability constants should be determined. The stability constant should be measured at the pH values where only one species of the molecule predominantly exists, i.e. for fisetin that would be pH 6, 9.5, 11.8 and 14. Based on the seven inputs; three pK_a values and four stability constants, it is possible to use the theoretical expression to estimate the overall stability constant as a function of pH.

To assist with the calculations, a graphical user interface was developed, and it can be found in Supplementary Information. The graphical user interface was developed in python using the 'tkinter' package, and is available in Supplementary Information as a python-file or an executable-file. It is a simple user interface, which allows the user to plot the overall stability constant as a function of pH (Fig. 9). The user chooses the number of pK_a values in a dropdown menu, and a number of entry fields are available according to the inputs needed to perform the calculations. The function is plotted based on the user's input. The plot of the overall stability constant as a function of pH can be saved on the user's computer as a png- or tiff-file by pressing the save icon. By clicking on the graph, the exact coordinates will be displayed.

4. Conclusions

The interaction between ionizable guest molecules and neutral CDs is affected by the pH of the solution. During drug formulation, the stability constant is an important parameter in optimization of final formulation, and the boundaries of a formulation must be evaluated over a pH range. In this work, theoretical expressions for the overall stability constant for CD complexes as a function of pH were derived for cases where the guest molecule has multiple pK_a values. The theoretical expression was validated by comparing with empirical data sets found in the literature. The inputs in the theoretical expression are pK_a values and experimentally determined stability constants at specific pH values. A graphical user interface was developed to facilitate easy use of the theoretical expression.

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Figure legends

Scheme 1. Equilibrium diagram for CD interaction with ionizable guest molecule HD.

Scheme 2. Equilibrium diagram of interaction between CD and amphoteric guest with three ionization states

Scheme 3. Equilibrium diagram for interaction between CD and amphoteric guest molecule with four ionization states.

Figure 1. Distribution of species for an amphoteric molecule with three ionization states; DH^+, D and D^-. The distribution depends on the 2 pK_a values; (A) 3.0 and 9.0, (B) 4.5 and 6.0.

Figure 2. Distribution of species for a molecule with five ionization states. The distribution depends on the 4 pK_a values: 2.0, 5.5, 9.0 and 12.5 (A) or 3.5, 4.5, 5.0 and 8.0 (B).

Figure 3. Overall stability constants pH profile for acidic (A, B), basic (C, D) and amphoteric (E, F) guest molecule with the same set of 3 pK_a values; 3.0, 7.0 and 11.0 (A, C, E) or 4.0, 5.0 and 6.0 (B, D, F). The empirical stability constants were set to 12000 M^{-1}, 9000 M^{-1}, 6000 M^{-1} and 3000 M^{-1}.

Figure 4. Distribution of species in sildenafil, when the pK_a values are 7.10 and 9.84.

Figure 5. The apparent stability constant (M^{-1}) as a function of pH based on the theoretical expression and empirical data for sildenafil and β-CD (Al Omari et al., 2006). K_1 = 17 M^{-1}, K_2 = 150 M^{-1}, K_3 = 42 M^{-1}, pK_{a1} = 7.10, pK_{a2} = 9.84.

Figure 6. Fitting of the apparent stability constant (M^{-1}) as a function of pH based on empirical data for sildenafil and β-CD (Al Omari et al., 2006). The pK_a values for sildenafil 7.10 and 9.84 was used.

Figure 7. Distribution of species for fisetin based on the pK_a values 8.87, 10.31 and 13.20.

Figure 8. The apparent stability constant (M^{-1}) as a function of pH for fisetin based on the pK_a values 8.87 and 10.31, and K_1 = 900 M^{-1}, K_2 = 702 M^{-1} and K_3 = 240 M^{-1}. This is a predicted curve based on knowing K_1 and K_3, and estimating a suitable value for K_2.

Figure 9. Graphical user interface developed for easy application of the theoretical expression described in this work.