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Characterization of drug interactions with soluble β-cyclodextrin by high-performance affinity chromatography

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Abstract

This study examined the use of an immobilized human serum albumin (HSA) column to study solution-phase reactions between drugs and β-cyclodextrin (β-CD). Chromatographic equations were developed to characterize the binding of chemicals to a soluble ligand (β-CD) in the presence of an independent immobilized ligand (HSA). Situations considered included the presence of both a homogeneous and heterogeneous immobilized ligand, as well as complex interactions between the chemical of interest and soluble ligand. Three drugs (warfarin, tamoxifen, and phenytoin) were examined by this approach. This method involved injecting a small amount of each drug onto an HSA column in the presence of various concentrations of β-CD in the mobile phase. By measuring the change in the drug's retention factor as the concentration of β-CD was varied, it was possible to determine the stability constant between the injected drug and β-CD. With this approach, warfarin and β-CD were found to have 1:1 interactions with a stability constant of $5.2 \times 10^2\ M^{-1}$ at 37 °C and pH 7.4, a result in close agreement with previous literature values. Tamoxifen and phenytoin were also found to have 1:1 interactions with β-CD and had stability constants of $0.9–1.2 \times 10^4$ and $6–9 \times 10^2\ M^{-1}$, respectively. With these latter solutes, the effects of secondary binding to the chromatographic support had to be considered. The theory and methods described in this report are not limited to these drugs and β-CD but can be applied to other analytes and soluble ligands.

Keywords: Affinity sorbents; Stability constants; Pharmaceutical analysis; Warfarin; Tamoxifen; Phenytoin

1. Introduction

Cyclodextrins have been widely used in the pharmaceutical industry for improving drug stability, dissolution rates, and bioavailability. To predict the effects of cyclodextrins on these properties, it is important to know the strength with which these agents bind to drugs. Many approaches have been used to examine this, including spectroscopic methods, electrochemical techniques, calorimetry, kinetic methods, and competition assays [1].

One technique used for this purpose is the Hummel–Dreyer method [2–4]. This typically uses a size-exclusion column onto which a cyclodextrin is injected in the presence of various mobile phase concentrations of a drug. As the drug binds to the cyclodextrin, a vacancy peak forms due to depletion of the non-complexed form of the drug from the mobile phase. The area of this vacancy peak and the mobile phase concentration of the drug are then used to determine the stability constant for the drug–cyclodexrin complex. Although this method is simple to perform, it requires good resolution between the vacancy peak and peak due to the injected cyclodextrin. This can be a problem when working with size-exclusion columns due to their limited retention range. Poor resolution can also result from peak tailing, such as caused by secondary interactions between the drug or cyclodextrin with the column.

Other methods used for studying the binding of drugs to cyclodextrins have included reversed-phase liquid chromatography (RPLC), normal-phase liquid chromatography (NPLC), cyclodextrin-bonded phase liquid chromatography, and thin-layer chromatography (TLC) [5–9]. One problem with RPLC is that this generally requires the use of an organic solvent in the mobile phase, causing possible deviations from the binding results expected under physiological conditions [5,6]. This problem can be minimized by using aqueous solvents in the other listed chromatographic methods; however, these methods tend to give low retention [7–9] in such binding studies, making it difficult to measure small changes in drug–cyclodextrin interactions as the chromatographic conditions are varied.

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Another approach that has been used for such work is affinity capillary electrophoresis (ACE) [10–13]. ACE can also be used under aqueous conditions but can be difficult to employ with drugs that have weak binding to cyclodextrins, since the concentration of cyclodextrin needed to produce a measurable shift may exceed the solubility of this agent [10]. In addition, appropriate precautions must be taken in ACE to account for changes in the running buffer viscosity as the cyclodextrin's concentration is altered [10]. Furthermore, ACE cannot be used for neutral drugs if their binding is being studied with an uncharged cyclodextrin.

Recent work with immobilized human serum albumin (HSA) columns has suggested an alternative approach for measuring stability constants between drugs and cyclodextrins [14–16]. In these previous studies, β-cyclodextrin (β-CD) was used as a solubilizing agent to examine the binding of low solubility drugs to immobilized HSA. This was made possible by the fact that β-CD and HSA act as independent binding agents that have no significant interactions with one another [14,17]. In these reports, the retention factor (k) for a drug injected onto an HSA column was measured in the presence of several concentrations of a competing agent while also varying the concentrations of β-CD used as a solubilizing agent. Plots of $1/k$ versus the competing agent concentration were then made at each level of solubilizing agent, providing the association constant for HSA with the injected drug. However, it was also noted that this approach might be adapted for examining the binding of drugs directly with a solubilizing agent like β-CD [14–16].

This study will expand upon this earlier work and use immobilized HSA to look at drug interactions with β-CD in solution. As shown in Fig. 1, this will make use of an HSA column onto which various concentrations of β-CD will be applied as a mobile phase additive. The drug of interest will then be injected and its retention factor measured, thus giving information on the stoichiometry and stability constant for the drug–cyclodextrin complex. The theory of this approach will be considered and its use will be evaluated by using warfarin, tamoxifen and phenytoin as model drugs. The potential advantages of this technique will be discussed, as well as its possible use with other solute–ligand systems.

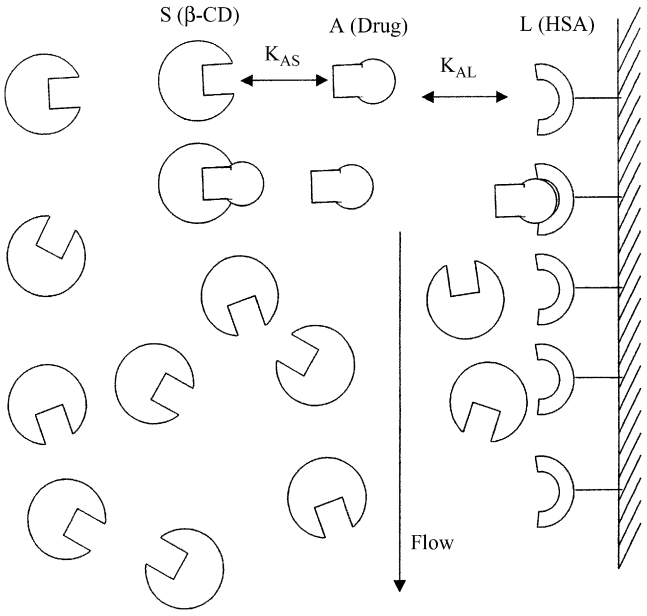

Fig. 1. General reactions involved in the determination of stability constants for a drug or other solute (A) with a soluble ligand (S) in the presence of a column containing an immobilized ligand (L).

2. Theory

The general approach used in this study involved the competition of two independent ligands for the same injected solute. The first of these ligands is attached to a chromatographic support, while the second is present in a soluble form in the mobile phase (see Fig. 1). In this report, the immobilized ligand was HSA and the soluble ligand was β-CD. When a drug or other small solute is applied to such a system, it can have interactions with either the soluble or immobilized ligand. As binding to the soluble ligand increases, the injected substance will spend less time in the stationary phase and be less retained on the column. Thus, by looking at how this chemical's retention changes as a function of the concentration of soluble ligand, it is possible to determine the stability constant for this interaction. A similar approach has previously been used in pseudo-phase liquid chromatography to examine the interactions of solutes with micelles and cyclodextrins on more traditional liquid chromatographic columns [7].

2.1. Analytes with 1:1 interactions with the soluble and immobilized ligands

The simplest case for this type of experiment is one in which a 1:1 complex is formed between the injected analyte (A) and soluble ligand (S), as well as between the analyte and immobilized ligand (L). This is represented by the following reactions:

$$A + L \overset{K_{AL}}{\rightleftharpoons} A\text{–}L \quad (1)$$

$$A + S \overset{K_{AS}}{\rightleftharpoons} A\text{–}S \quad (2)$$

where A–L and A–S are the complexes formed between the analyte and L or S. The terms K_{AL} and K_{AS} are the stability constants (or association equilibrium constants) for these interactions. In this model, it is assumed that the soluble ligand is not retained by the immobilized ligand or that the presence of such retention does not affect the binding of the analyte with the soluble ligand. It is also assumed that there is no binding between L and the complex that forms between A and S.

For this reaction scheme, the following relationship describes how the retention factor for the analyte will change

as the concentration of free solubilizing agent, [S], is varied in the mobile phase (see Appendix for derivation).

$$\frac{1}{k} = \frac{1}{K_{AL}[L]} + \frac{K_{AS}[S]}{K_{AL}[L]} \quad (3)$$

The term [L] in this equation represents the effective concentration of the non-complexed form of the immobilized ligand. According to this equation, a plot of $1/k$ versus [S] should give linear relationship, where the ratio of the slope to intercept gives the stability constant K_{AS} for the analyte with the solubilizing agent. An equivalent expression has been derived earlier for use in pseudo-phase liquid chromatography [7].

One assumption made in Eq. (3) is that the analyte is present at a much lower concentration than the solubilizing agent or immobilized ligand (i.e., linear elution conditions are present). Under these conditions, the total concentration of solubilizing agent can be used in place of [S], since the amount of solubilizing agent in the complexed form (A–S) is small compared to the total amount of this agent. Another assumption made in Eq. (3) is that the analyte has only 1:1 interactions with L and S. More advanced equations that allow for different reaction stoichiometries will be given in the following section.

2.2. Analytes with multiple bindings sites on the soluble or immobilized ligands

Eq. (3) can be extended to a more general case in which the analyte has multiple binding sites on either L or S. For instance, consider the case where there is more than one binding site on L for A or there is more than one type of immobilized ligand for A. If these ligands act independently, they can be said to have association equilibrium constants $K_{AL1}, K_{AL2}, \ldots, K_{ALm}$ and effective concentrations of $[L_1]$, $[L_2]$, $\ldots$, $[L_m]$. Under these conditions, Eq. (3) converts to the form shown below:

$$\frac{1}{k} = \frac{1}{\sum_{i=1}^{m} K_{ALi}[L_i]} + \frac{K_{AS}[S]}{\sum_{i=1}^{m} K_{ALi}[L_i]} \quad (4)$$

It is still assumed in this relationship that the experiments are being performed under linear elution conditions and the solute has 1:1 interactions with the soluble ligand. Like Eq. (3), the above expression indicates that a plot of $1/k$ versus [S] will give a linear relationship, with the ratio of the slope to the intercept providing the value for K_{AS}. The only difference between Eqs. (3) and (4) is that the latter equation now includes a denominator which represents the sum of association constants and concentrations for all types of immobilized ligands in the column.

This model can also be modified to include systems in which the analyte has interactions with several soluble ligands. For example, in a system where "n" soluble ligands bind to the same analyte, Eq. (4) takes the form shown below (see Appendix for derivation).

$$\frac{1}{k} = \frac{1}{\sum_{i=1}^{m} K_{ALi}[L_i]} + \frac{K_{AS}[S]^n}{\sum_{i=1}^{m} K_{ALi}[L_i]} \quad (5)$$

Once again, the value of K_{AS} can be determined, as is found by now plotting $1/k$ versus $[S]^n$ and taking the ratio of slope to the intercept. If several complexes with different stoichiometries coexist, then a non-integer value for n will be obtained or deviations from linearity will be noted when using Eq. (5).

2.3. Corrections for secondary binding

For some systems secondary interactions between an injected analyte and the column may be present. If these sites have the same binding stoichimetry with the analyte as L, they can be included as a subgroup of "ligand" sites in Eqs. (4) and (5). In this case, these secondary sites would be represented by one of the K_{ALi} $[L_i]$ terms in the denominator of these expressions. A linear response to Eqs. (4) and (5) would still be expected in this situation, with the value of K_{AS} again being obtained by taking the ratio of the slope to the intercept for a plot of $1/k$ versus [S].

A more complex situation arises if the secondary interactions do not have the same stoichiometry as L when binding with the analyte. In this situation, the value of K_{AS} can be obtained by performing two sets of experiments in which the analyte is injected in the presence of the solubilizing agent onto both the immobilized ligand column and a control column of identical size with the same support but no immobilized ligand. A similar approach has been used in other zonal elution studies to correct for multi-site interactions [18]. If the retention due to secondary interactions is assumed to be the same for the two columns, then the retention factor measured for the control column (k_s) can be simply subtracted from the solute's total retention factor (k_{tot}) on the immobilized ligand column, as shown in Eq. (6).

$$\frac{1}{k_{tot} - k_s} = \frac{1}{\sum_{i=1}^{m} K_{ALi}[L_i]} + \frac{K_{AS}[S]^n}{\sum_{i=1}^{m} K_{ALi}[L_i]} \quad (6)$$

If it is not assumed that k_s is the same between the control column and immobilized ligand column, another route for estimating this is to use least-squares regression to estimate k_s from a fit of Eq. (6) to the experimental data obtained on the immobilized ligand column. This is similar to the method described in ref. [18] as a means of correcting for non-competitive interactions.

3. Materials and methods

3.1. Reagents

The HSA (99% pure, essentially fatty acid free), racemic warfarin (>98% pure), phenytoin (99% pure), tamoxifen ([*Z*]-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-

1-butene), and β-CD (>98% pure, containing 12% (w/w), water) were from Sigma (St. Louis, MO, USA). Nucleosil Si-1000 silica (5 μm particle size, 1000 D pore size) and Nucleosil Si-300 silica (7 μm particle size, 300 D pore size) were from P.J. Cobert (St. Louis, MO, USA). The reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). All solutions were prepared with water obtained from a NANOpure water system (Barnstead, Dubuque, IA, USA).

3.2. Apparatus

The chromatographic system consisted of a Jasco PU980 pump (Easton, MD, USA), a ThermoSeparation AS3000 autosampler (Riviera Beach, FL, USA), an Alltech water jacket (Deerfield, IL, USA), and a Milton Roy SM3100 UV-Vis absorbance detector (Riviera Beach, FL, USA). The water jacket temperature was controlled with water circulated by an Isotemp 9100 water bath (Fisher Scientific, Pittsburgh, PA, USA); this water was used to control the temperature of the HSA and control columns and to preheat the mobile phases before they passed through these columns. The chromatographic data were collected using an in-house program written in Labview (National Instruments, Austin, TX, USA).

3.3. Methods

The Nucleosil-1000 and Nucleosil-300 silica were converted into a diol-bonded form using a previously published method [19]. The resulting diol coverage of the Nucleosil Si-1000 was found to be 37±1 μmol/g silica (±S.D.) and the coverage for the Nucleosil Si-300 silica was 290±20 μmol/g silica, as determined in triplicate by an iodometric capillary electrophoresis assay [20].

HSA was immobilized onto each diol-bonded support by the Schiff base method [21]. This first involved the conversion of the diol-bonded supports into an aldehyde-activated form. For the preparation of HSA Nucleosil Si-1000 silica, 0.4 g/ml aldehyde silica was mixed with 40 mg/ml HSA and allowed to react with shaking at 4 °C for 66 h. For the preparation of HSA Nucleosil Si-300 silica, 1.0 g/ml aldehyde silica and 150 mg/ml HSA were mixed and shaken for 6 days at 4 °C. After immobilization, each batch of HSA silica was centrifuged and washed three times with pH 7.4, 0.067 M potassium phosphate buffer. A control support was prepared in a similar manner by taking the diol-bonded silica through the entire immobilization procedure but with no HSA being added. Both the HSA and control supports were stored in pH 7.4, 0.067 M potassium phosphate buffer at 4 °C until use.

The immobilized HSA and control supports made from Nucleosil Si-1000 were downward slurry-packed into separate 5 cm × 2.1 mm i.d. stainless steel columns. The supports prepared from Nucleosil Si-300 were similarly packed into separate 5 cm × 4.6 mm i.d. stainless steel columns. All these columns were packed at 3500 psi using pH 7.4, 0.067 M potassium phosphate buffer as the packing solvent. The protein content of the HSA support prepared from Nucleosil Si-1000 was 10.9 ± 0.1 mg HSA/g silica and the protein content of the HSA support made from Nucleosil Si-300 was 54 ± 5 mg HSA/g silica, as determined in replicate by a BCA protein assay [22].

The zonal elution experiments in the warfarin and tamoxifen studies were typically performed at a flow rate of 0.3 ml/min. For the phenytoin studies, a flow rate of 1.0 ml/min was used. No shifts in the retention factors for any of these analytes were noted when the small changes were made in the flow rate in these experiments, indicating that suitably fast association and dissociation kinetics were present for the determination of equilibrium constants from the true central moment for each peak.

The typical samples used in this study consisted of 20 μl injections of 0.25 μM warfarin, 0.2 μM tamoxifen, or 0.04 μM phenytoin. Each of analytes was dissolved in the same mobile phase as was being used in the zonal elution experiment. These particular sample concentrations were selected based on their ability to provide measurable peaks while also giving retention factors that did not vary with a change in analyte concentration, thus indicating the presence of linear elution conditions. An example of such a study is illustrated in Fig. 2.

The mobile phase for the zonal elution studies was prepared using pH 7.4, 0.067 M potassium phosphate buffer, to which was added 0–4.4 mM β-CD. Due to their higher solubility in the pH 7.4 potassium phosphate buffer, it was also possible to inject warfarin and phenytoin directly into mobile phases that did not contain β-CD. However, this was not possible for tamoxifen, whose low solubility prevented it from being used in detectable amounts unless β-CD was added as a solubilizing agent. For this drug, the lowest

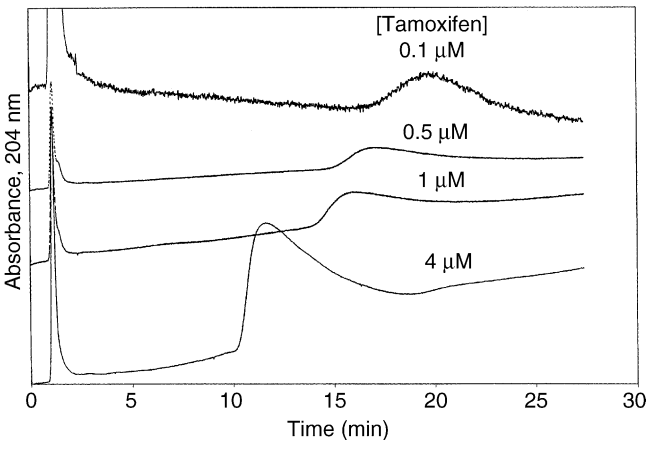

Fig. 2. Effect of varying the injection concentration of tamoxifen on the retention of this compound on an HSA column. The stock solution contained 4 μM tamoxifen dissolved in a pH 7.4, 0.067 M potassium phosphate buffer containing 2.2 mM β-CD. The mobile phase also contained 2.2 mM β-CD in pH 7.4, 0.067 M potassium phosphate buffer. All other analyte solutions were prepared by mixing the stock tamoxifen solution with the mobile phase. Other conditions are given in the text.

mobile phase concentration of β-CD that could be used was 0.88 mM.

The analytes were detected at a wavelengths of 204 nm (warfarin and tamoxifen) or 205 nm (phenytoin). Duplicate or triplicate injections were made under each set of experimental conditions. The column void time was determined by injecting 20 μl of 0.27 mM sodium nitrate. The system void time was determined by injecting sodium nitrate in the presence of a zero dead volume union instead of a column and was used to correct all retention times and void times used in the calculation of retention factors. The chromatographic data were collected at a rate of 1 point/s for warfarin and tamoxifen and 10 points/s for phenytoin, which had lower retention than the other two analytes. The pressure drop across the HSA and control columns was less than 10 kg/cm^2 (140 psi) during all these studies. The retention time for each peak was determined after baseline correction by using either moment analysis or the $B/A_{0.5}$ method [23].

4. Results and discussion

4.1. Binding of warfarin to β-CD

The first drug examined in this study was warfarin (Fig. 3). Warfarin is a common anticoagulant. It exists in two enantiomeric forms, (*R*)- and (*S*)-warfarin, which were used in this study as a racemic mixture. Warfarin is a weak acid with a pK_a of about 5. It also has a relatively low solubility in an aqueous solvent at pH 7.4, with an upper solubility of approximately 58.4 μM [24]. One way this solubility can be increased is to add β-CD as a complexing agent for warfarin. Previous studies have used a variety of methods to examine the binding of warfarin with cyclodextrins, including fluorimetric measurements [24] and HPLC [4]. In these studies, the results have indicated that only 1:1 complexes form between warfarin and β-CD or its derivatives.

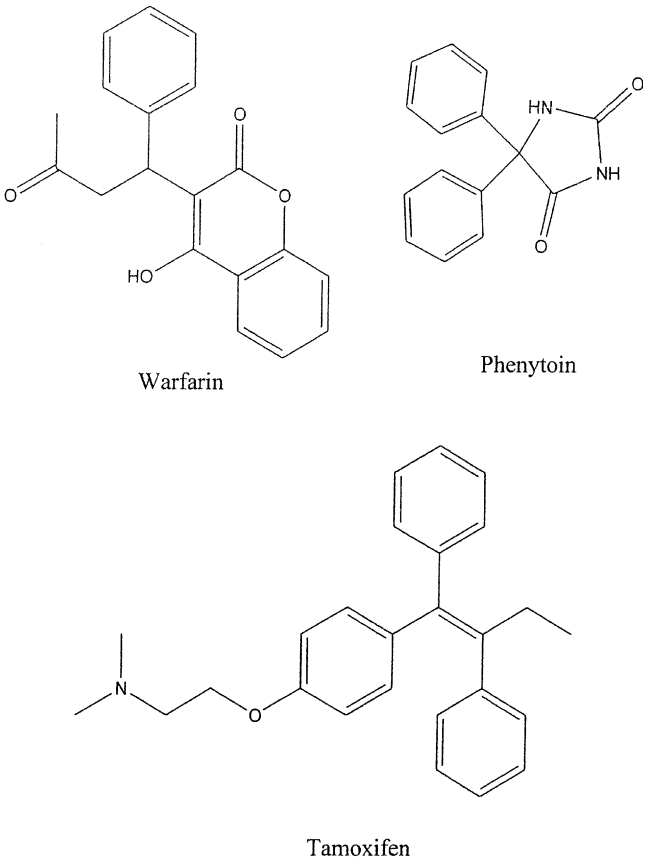

Fig. 3. Structures of warfarin, tamoxifen and phenytoin.

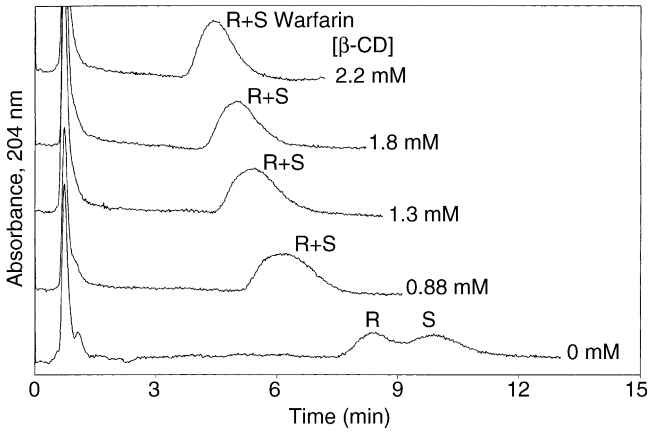

Fig. 4. Chromatograms obtained for a racemic mixture of (*R*)- and (*S*)-warfarin injected onto an immobilized HSA column in the presence of various concentrations of β-CD in the mobile phase. Other conditions are given in the text.

Fig. 4 shows the results obtained when the retention of (*R*)- and (*S*)-warfarin was examined on an HSA column in the presence of various concentrations of β-CD. As can be seen from this figure, the retention of both warfarin enantiomers decreased as the concentration of β-CD increased. The corresponding retention factors that were measured are shown in Table 1. As reported in previous work, the elution of warfarin on the control column was almost the same as the column void time [20,25]. In this particular case, the control column gave retention factors for warfarin that ranged from 0.07 to 0.23, which were less than 2.7% of the retention factors noted for warfarin on the immobilized HSA column. Thus, with this analyte it was possible to ignore

Table 1
Retention factors for racemic warfarin on an immobilized HSA column in the presence of various mobile phase concentrations of β-CD[a]

[β-CD] (μM)	*k*
0	19.8 ± 0.2
0.88	12.7 ± 0.2
1.3	11.1 ± 0.1
1.8	10.2 ± 0.1
2.2	8.9 ± 0.1

[a] The range following each result represents ±1S.D. All retention factors were measured in pH 7.4, 0.067 M potassium phosphate buffer at 37 °C.

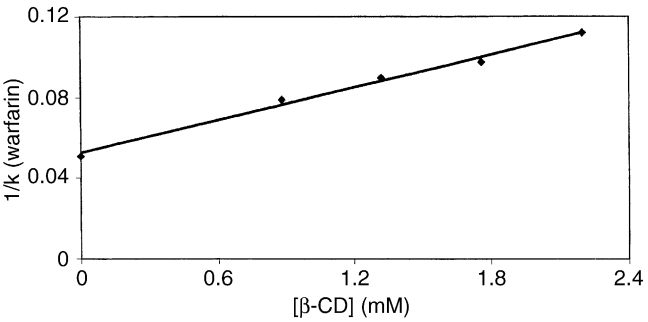

Fig. 5. Plot of 1/*k* vs. [β-CD] for racemic warfarin injected onto an immobilized HSA column. The slope and intercept were 0.027 ± 0.001 and 0.052 ± 0.002, respectively. Each data point represents the average of two or more injections. Other parameters are given in the text.

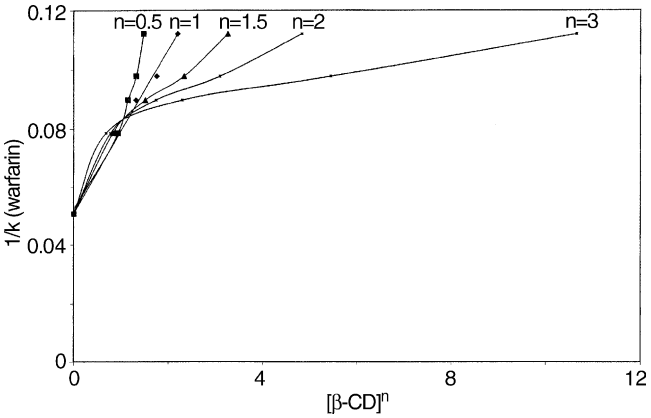

Fig. 6. Plots of 1/*k* vs. $[\beta\text{-CD}]^n$ for warfarin injected onto an HSA column with $n = 0.5$, 1.0, 1.5, 2 or 3. Each data point represents the average of two or more injections.

the contribution of secondary binding to the column when determining warfarin's stability constant with β-CD.

It is interesting to note in Fig. 4 that when no β-CD was present two separate peaks for (*R*)- and (*S*)-warfarin were obtained on the HSA column. However, when even 0.88 mM β-CD was present, little or no resolution was obtained between these two peaks. As a result, the retention factors shown in Table 1 are the averages for these enantiomers. This includes the result given for warfarin in the presence of no β-CD, which was simply obtained by averaging the separate retention factors measured for (*R*)- and (*S*)-warfarin in the presence of the pH 7.4 potassium phosphate buffer.

Based on the retention factors in Table 1, a plot of 1/*k* versus [β-CD] was made according to Eq. (3). The result is shown in Fig. 5. This gave a linear relationship (correlation coefficient = 0.998 for five data points), which suggested that 1:1 binding was occurring between β-CD and each of the warfarin enantiomers. Based on the slope and intercept of this graph, the average stability constant for (*R*)- and (*S*)-warfarin with β-CD was found to be $5.2 \pm 0.3 \times 10^2\ M^{-1}$ at pH 7.4 and 37 °C. This was statistically identical to the value of $5.4 \pm 0.2 \times 10^2\ M^{-1}$ that has been obtained by fluorimetric measurements [24] and the result of $5.2 \pm 0.3 \times 10^2\ M^{-1}$ that has been reported through the use of an alternative HPLC method [4].

Using the measured value for K_{AS} along with the known concentration of injected warfarin, [A], it was possible to estimate the maximum value for K_{AS} [A] in these experiments. This gave a result of 1.1×10^{-4} under the conditions used in this study. This was much less than one, confirming that linear elution conditions were present, since C_S was approximately equal to [S]. Thus, it was valid to use the total concentration of β-CD in place of its non-complexed concentration in the preparation of Fig. 5 and in the analysis of this figure according to Eq. (3).

As indicated by Eq. (3), the intercept in Fig. 5 should be equal to $1/(K_{AL}\ [L])$. This, in turn, gave a value for K_{AL} [L] of 19.2 ± 0.7. Using this result, an estimate was obtained for K_{AL} by using an independent value for [L]. For instance, the support used in Fig. 5 was determined to contain 10.9 ± 0.1 mg HSA/g silica and had a packing density within the column of 0.45 mg/ml. This meant the total effective concentration of HSA in this column was 74 ± 1 μM. If it is assumed that most of this HSA was active, then the value of K_{AL} for warfarin with the immobilized HSA would be $2.6 \pm 0.1 \times 10^5\ M^{-1}$ at pH 7.4 and 37 °C. This shows good agreement with previous association constants reported for warfarin with immobilized HSA at the same temperature and pH, where values of 2.1×10^5, 2.6×10^5, and $2.4 \times 10^5\ M^{-1}$ have been reported for (*R*)-, (*S*)-warfarin, and racemic warfarin, respectively [25].

It was noted in the analysis of these data that only a plot of 1/*k* versus [β-CD] gave a good linear relationship, as predicted by Eqs. (3)–(5) for a system with 1:1 interactions (i.e., where $n = 1$ in Eq. (5)). The use of integers greater than one in Eq. (5) gave curved lines with a poor fit to the data, as shown in Fig. 6. The same was true for fits that involved non-integer values of n. All these results supported the conclusion that a 1:1 complex was the main product formed between warfarin and β-CD.

4.2. Binding of tamoxifen to β-CD

Tamoxifen was the second drug considered in this study. Tamoxifen is an anti-estrogen drug used for the treatment of breast cancer. This is used as the *trans*-isomer of the triphenylethylene derivative, as shown in Fig. 3. The corresponding *cis*-isomer has no clinical applications and is usually not present in preparations of this drug. Tamoxifen is a basic drug with a pK_a of 8.85. Its solubility in water is roughly 80 μM, with a much lower solubility being seen in pH 7.4 buffer. To increase this solubility, this drug is often used in the form tamoxifen citrate. Although β-CD is seldom seen in tamoxifen formulations, this agent has been used in previous reports to help solubilize tamoxifen for measurements of its binding to HSA [14–16].

The low solubility of tamoxifen in pH 7.4 buffer required the use of β-CD as a mobile phase additive in the tamoxifen

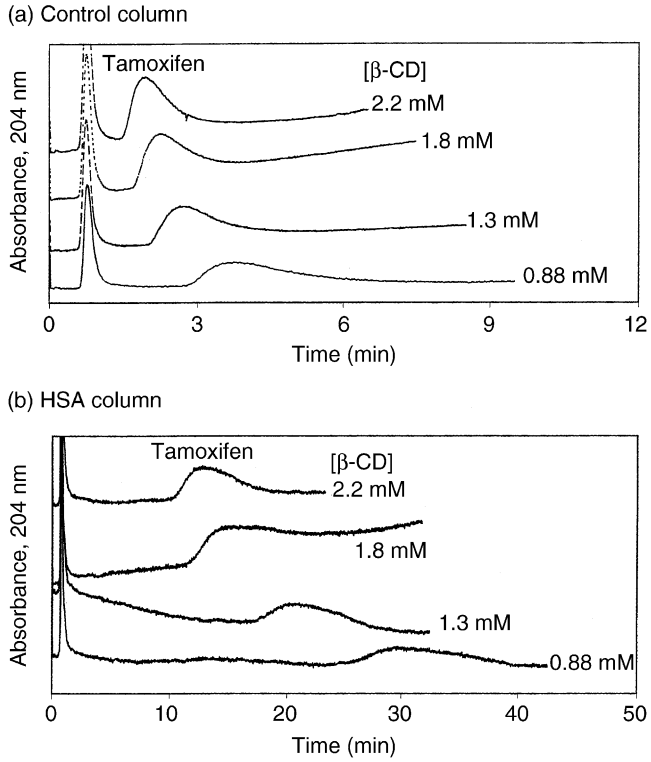

Fig. 7. Chromatograms obtained for tamoxifen injected onto (a) a control column with no HSA and (b) a HSA column in the presence of various mobile phase concentrations of β-CD. Other conditions are given in the text.

experiments. Another complication of working with tamoxifen was its moderate retention on the column support even when no HSA was present. This is demonstrated in Fig. 7 and in Table 2, where a control column containing no protein gave retention factors of 8.34–3.57 for tamoxifen in the presence of 0.88–2.2 mM β-CD. Even higher retention would be expected for tamoxifen on such a column at lower β-CD concentrations. Based on the data in Table 2, it was estimated that this secondary binding accounted for up to 13% of the total retention seen for tamoxifen on the HSA column. Thus, this binding could not be ignored when HSA columns were used to examine the binding of tamoxifen with soluble β-CD.

Table 2
Retention factors for tamoxifen on a control column ($k_{Control}$) and HSA column (k_{HSA}) in the presence of various mobile phase concentrations of β-CD[a]

[β-CD] (μM)	$k_{Control}$	k_{HSA}	$k_{HSA} - k_{Control}$
0.88	8.34 ± 0.04	71.3 ± 1.0	62.9 ± 1.0
1.3	5.98 ± 0.07	49.1 ± 0.4	43.1 ± 0.4
1.8	4.54 ± 0.10	37.6 ± 0.7	33.0 ± 0.7
2.2	3.57 ± 0.01	30.2 ± 0.3	26.6 ± 0.3

[a] The range following each result represents ±1S.D. All retention factors were measured in pH 7.4, 0.067 M potassium phosphate buffer at 37 °C. The terms $k_{Control}$ and k_{HSA} in this table are equivalent in this case to the more general terms k_s and k_{tot} in Eqs. (6), (A.19), and (A.20).

In looking at Eq. (3) and its derivation, it can be seen that no restrictions are given on the type of immobilized ligand that must be present. This means that this equation should work equally well for strong interactions with a well-defined ligand (e.g., the binding of tamoxifen with HSA) or for weaker and less defined sites (e.g., secondary interactions between tamoxifen and the support). Thus, Eq. (3) was used to determine how the retention of tamoxifen on the control column was affected by the presence of β-CD. These results were then used to correct for the secondary binding of tamoxifen to the support in the HSA column. Previous studies have used the same general approach to examine the binding of aromatic compounds with β-CD in the presence of a Supelcosil-CN column [9], a material similar in polarity to the control column used in this report.

Fig. 8a shows the results for the control column when the data in Table 2 were used to make a plot of 1/k versus [β-CD]. The result was a linear relationship with a correlation coefficient of 0.999 over four points. This indicated that the binding of tamoxifen to the control support could be described by a simple 1:1 interaction model. From the intercept and the slope of this plot, the stability constant for tamoxifen with the β-CD was found to be $1.2 \pm 0.9 \times 10^4\ M^{-1}$. From

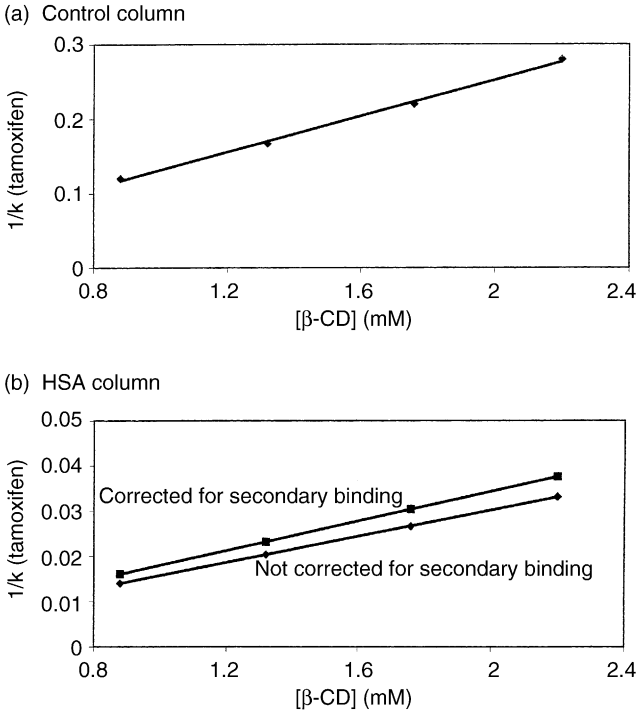

Fig. 8. Plots of 1/k vs. [β-CD] for tamoxifen injected onto (a) a control column containing no HSA and (b) a column containing the same support with immobilized HSA. Each data point represents the average of at least two injections. The best-fit slopes and intercepts for these plots were 0.121 ± 0.005 and 0.010 ± 0.007 for the control column, $1.44 \pm 0.01 \times 10^{-2}$ and $1.3 \pm 0.1 \times 10^{-3}$ for the HSA column with no correction for secondary binding, and $1.64 \pm 0.01 \times 10^{-3}$ and $1.5 \pm 0.1 \times 10^{-3}$ for the HSA column after correcting for secondary binding. Other information on these plots can be found in the text.

the intercept of this plot, the product K_{AL} [L] was estimated to be 100 ± 70, thus providing information on the relative affinity of the support for tamoxifen. It is also possible to obtain K_{AL} between tamoxifen and this support by using this information along with independent estimates of [L]. For instance, it was determined that 16.7 ± 0.5 mM spacer groups and 18.9 ± 0.5 mM alcohol groups were present in this column, as created during the production of the control support. If either of these groups were responsible for the secondary binding of tamoxifen, then the association constant for these interactions would be approximately $6 \pm 4 \times 10^3\ M^{-1}$. Although this represents relatively strong secondary interactions, it should be pointed out that the relatively large imprecision of this particular binding constant (due to the need to work at fairly high cyclodextrin concentrations with tamoxifen) makes this only a rough approximation and not an exact value.

The results obtained for tamoxifen on the HSA column are shown in Fig. 8b before and after a correction had been made for the secondary binding of tamoxifen to the support. This correction was made by assuming the HSA and support acted independently in their binding to tamoxifen and were additive in terms of the overall retention that was measured for this drug. It was also assumed that the number of secondary sites was essentially the same on the control and HSA columns. This made it possible to obtain the corrected retention factors by simply subtracting the k values for tamoxifen on the control column from those measured for tamoxifen on the HSA column at the same concentrations of β-CD.

As is shown in Fig. 8b, both the corrected and non-corrected results for tamoxifen on the HSA gave good linear behavior when plotted according to Eq. (3), with correlation coefficients of 0.998 over four data points. In these plots, the data which had been corrected for secondary binding gave higher $1/k$ values (i.e., retention factors that were up to 13% lower) and a larger intercept. When a correction had been made for the secondary binding, the stability constant determined for tamoxifen with β-CD was found to be $1.1 \pm 0.1 \times 10^4\ M^{-1}$. Without this correction, the stability constant was estimated to be $0.97 \pm 0.03 \times 10^4\ M^{-1}$, a slightly lower but not significantly different result. In both cases, the product K_{AS} [A] was calculated to be much less than one (<0.02), indicating that the requirement for linear elution had been met and allowing use of the total concentration for β-CD in place of its non-complexed concentration in Fig. 8. Furthermore, the analysis of these data by Eq. (5) indicated that $n = 1$ gave the best-fit, supporting a model in which a 1:1 complex was forming between tamoxifen and β-CD.

As shown earlier for warfarin, it was possible to use the plots in Fig. 8 to get information on the binding of tamoxifen with the immobilized HSA. As stated earlier, the HSA column had an effective protein concentration of 74 ± 1 μM. If all of this HSA were active, then the combination of this value with the intercept from Fig. 8b would give a binding constant for tamoxifen with the immobilized HSA of $9.0 \pm 0.6 \times 10^6\ M^{-1}$ after correcting for secondary binding. This value is similar in size to that reported for immobilized HSA with clomiphene, a drug closely-related to tamoxifen, which has association constants of $7.5 \pm 0.2 \times 10^6$ and $1.3 \pm 0.2 \times 10^6\ M^{-1}$ for its *cis*- and *trans*-isomers [14,15].

Table 3
Retention factors for phenytoin on a control column ($k_{Control}$) and HSA column (k_{HSA}) in the presence of various mobile phase concentrations of β-CD[a]

[β-CD] (μM)	$k_{Control}$	k_{HSA}	$k_{HSA} - k_{Control}$
0	1.56 ± 0.01	4.45 ± 0.01	2.89 ± 0.02
1.8	0.74 ± 0.01	2.00 ± 0.01	1.26 ± 0.02
2.6	0.56 ± 0.01	1.57 ± 0.01	1.01 ± 0.01
3.5	0.49 ± 0.01	1.26 ± 0.01	0.77 ± 0.01
4.4	0.41 ± 0.01	1.06 ± 0.01	0.65 ± 0.01

[a] The range following each result represents ±1S.D. All retention factors were measured in pH 7.4, 0.067 M potassium phosphate buffer at 37 °C. The terms $k_{Control}$ and k_{HSA} in this table are equivalent in this case to the more general terms k_s and k_{tot} in Eqs. (6), (A.19), and (A.20).

4.3. Binding of phenytoin to β-CD

The third drug considered in this report was phenytoin (See Fig. 3). Phenytoin is widely used as an anti-convulsant for the treatment of seizures. It is an achiral substance and a weak acid with a pK_a of 8.3. The solubility of phenytoin in aqueous solutions at physiological pH has been reported to be 70–105 μM [26–28]. At lower pH values, as may be encountered in the gastrointestinal tract, this solubility decreases and can affects this drug's absorption. As a result, cyclodextrins are often added to phenytoin preparations to increase its solubility. One of these agents is β-CD, which has been reported to form a 1:1 complex with phenytoin [29].

Preliminary studies performed with phenytoin indicated it was similar to tamoxifen in that it gave a significant amount of secondary binding to the support within the HSA column. This is demonstrated by the retention factors in Table 3. Although the retention factors seen for phenytoin on the control column were much lower than those noted for tamoxifen, this drug also had lower retention on the HSA column. As a result, the relative contribution of its secondary binding to the overall retention was larger, making up 35–40% of the HSA column's total retention for phenytoin.

Using the data in Table 3, plots of $1/k$ versus [β-CD] were made for phenytoin on both the control and HSA columns (figures not shown). The best-fit slopes and intercepts for these plots were 0.40 ± 0.01 and 0.65 ± 0.03 for the control column, 0.16 ± 0.01 and 0.22 ± 0.01 for the HSA column with no correction for the secondary binding, and 0.27 ± 0.01 and 0.32 ± 0.03 for the HSA column with a correction for the secondary binding. For the HSA column, both the overall retention factors were employed (i.e., the uncorrected data) as well as values that had been adjusted for the retention factors seen on the con-

Table 4
Summary of stability and association constants measured for various drugs with β-CD and HSA or control columns[a]

Drug	Type of column	Stability constant with β-CD (M^{-1})	Association constant with stationary phase (M^{-1})
Racemic warfarin	HSA	$5.2 \pm 0.3 \times 10^2$	$2.6 \pm 0.1 \times 10^5$
Tamoxifen	Control	$1.2 \pm 0.9 \times 10^4$	$6 \pm 4 \times 10^3$
	HSA (not corrected for k_s)	$0.97 \pm 0.03 \times 10^4$	–
	HSA (corrected for k_s)	$1.1 \pm 0.1 \times 10^4$	$9.0 \pm 0.6 \times 10^6$
Phenytoin	Control	$6.2 \pm 0.3 \times 10^2$	11.7 ± 0.9
	HSA (not corrected for k_s)	$7.5 \pm 0.3 \times 10^2$	–
	HSA (corrected for k_s)	$8.4 \pm 0.8 \times 10^2$	$1.2 \pm 0.1 \times 10^4$

[a] The range following each result represents ±1S.D. All stability or association constants were measured in pH 7.4, 0.067 M potassium phosphate buffer at 37EC.

trol column. All plots gave linear behavior with correlation coefficients greater than 0.998 ($n = 5$). From the slope and intercept obtained with the control column, the stability constant for phenytoin with β-CD was determined to be $6.2 \pm 0.3 \times 10^2\ M^{-1}$ at pH 7.4 and 37 °C. The data obtained with the HSA column gave similar values of $8.4 \pm 0.8 \times 10^2$ and $7.5 \pm 0.3 \times 10^2\ M^{-1}$ for the plots made with and without a correction for secondary interactions. All of these results were in reasonable agreement with a stability constant of $850\ M^{-1}$ that has previously been reported for phenytoin with β-CD at pH 7.4 and 25 °C [30]. The use of Eq. (5) with this data showed that the best-fits were obtained with $n = 1$, indicating that a 1:1 complex was forming between phenytoin and the β-CD. In each case, the term $K_{AL}[A]$ was estimated to be less than 3.4×10^{-5}, confirming that linear elution conditions were present during these studies.

It was possible with Eq. (3) to obtain information on the binding of phenytoin with the immobilized HSA, as discussed for warfarin and tamoxifen. This was again made possible by using the intercepts of the plots of $1/k$ versus [β-CD], which should have been equal to $1/(K_{AL}\ [L])$. In these experiments, an HSA column was used that had a measured protein content of 54 ± 5 mg HSA/g silica, or an effective concentration of $3.7 \pm 0.3 \times 10^2$ μM in the HSA column. If it is assumed that all of this protein was active, this gives a binding constant for phenytoin to HSA of about $1.2 \pm 0.1 \times 10^4\ M^{-1}$ after correcting for secondary binding. This is consistent with a result $0.9 \times 10^4\ M^{-1}$ reported in Ref. [31] under similar conditions.

For the control column, it was also possible to determine K_{AL} [L] for the secondary interactions of phenytoin with the support. This column made use of a smaller pore size support than that used in the warfarin and tamoxifen studies (i.e., 300 Å versus 1000 Å), which gave it a higher surface area and higher initial diol content [21]. In this case, the estimated concentration of alcohol groups or alkane linkers in the column was 131 ± 8 mM. By combining this concentration with the intercept of the plot $1/k$ versus [β-CD] for the control column, the binding of phenytoin to this support was determined to have an association constant of $11.7 \pm 0.9\ M^{-1}$ at pH 7.4 and 37 °C.

5. Conclusions

This study examined the use of zonal elution and immobilized HSA columns in the measurement of binding constants between drugs and soluble β-CD. Table 4 summaries the stability constants that were measured in this report. These values show good agreement with those reported by other methods [4,14,15,24,30]. In each case a 1:1 complex was found to be the dominant product, which also agrees with previous observations made in the literature [4,24,29].

It was found that both the HSA columns and control columns could be used in these measurements for some drugs (i.e., tamoxifen and phenytoin). However, the larger retention of the HSA columns gave them better precision when estimating stability constants for soluble β-CD, as indicated by the smaller standard deviations for their results in Table 4. For instance, the use of an HSA column for tamoxifen provided a 10–30-fold more precise estimate for its stability constant with β-CD versus the value obtained with the control column. In the case of warfarin, the control column could not be used at all for such studies since it gave little or no retention for this analyte. The one disadvantage of using the HSA column is that it was necessary that the drug of interest have some interactions with this protein.

The results obtained for both the HSA and control columns gave a good fit to a 1:1 binding model when these were used to examine the retention of tamoxifen and phenytoin. This meant that the immobilized HSA and secondary sites could be treated simultaneously with Eq. (3) to provide a direct estimate of the stability constant between the drugs and β-CD. This approach gave statistically identical results to those obtained when the retention data for the HSA column were corrected for the retention of tamoxifen and phenytoin on the control column.

There are several advantages to the approach described in this report. Unlike the Hummel–Dreyer method, only one peak per analyte is present in the chromatogram, making it easier to obtain accurate and precise measurements of changes in peak behavior. Compared to RPLC, this method has the advantage of not requiring any organic

additive, which allows it to be used under aqueous conditions. Compared to NPLC, cyclodextrin columns, and TLC, the larger retention of the HSA column gives this method the ability to make more precise estimates of the binding constants for β-CD. In addition, this technique should have a broader range of usable additive concentrations than ACE, making it useful for the study of weak cyclodextrin complexes [7,10]. For instance, previous work has already demonstrated that stability constants as low as 10^1–10^2 M^{-1} can be analyzed by such an approach [9].

6. Nomenclature

A	analyte
[A]	concentration of analyte
A–S	complex of A with soluble ligand S
[A–S]	concentration of complex A–S
A–L	complex of A with immobilized ligand L
[A–L]	effective concentration of complex A–L (i.e., m_{AL}/V_M)
A–L_i	complex of A with immobilized ligand L_i
[A–L_i]	effective concentration of complex A–L_i (i.e., m_{ALi}/V_M)
A–L′	complex of A with secondary site L′
[A–L′]	effective concentration of complex A–L′ (i.e., $m_{AL'}/V_M$)
C_S	total concentration of soluble ligand
k	retention factor
k_L	retention factor due to binding of A to an immobilized ligand
k_s	retention factor due to secondary binding of A to the support
k_{tot}	total retention factor for A
K_{AL}	association constant between A and immobilized ligand L
K_{ALi}	association constant between A and immobilized ligand L_i
K_{AS}	association constant between A and soluble ligand S
L	immobilized ligand
[L]	effective concentration of immobilized ligand L (i.e., m_L/V_M)
n	number of soluble ligands S that bind to one analyte molecule
m	number of independent immobilized ligand sites for A
m_A	moles of analyte in the mobile phase
m_{AS}	moles of complex AS in the mobile phase
m_{AL}	moles of complex AL in the stationary phase
$m_{AL'}$	moles of A bound to secondary ligand L'
S	soluble ligand
[S]	concentration of non-complexed soluble ligand
V_M	column void volume

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Appendix A. Derivation of Eq. (3) for analytes and ligands with 1:1 interactions

By definition, the retention factor (k) for an injected analyte (A) should be equal to the moles of A present in the stationary phase versus the moles in the mobile phase at equilibrium. For a system in which A binds to an immobilized ligand (L) and a soluble ligand (S) with 1:1 interactions, the following relationships show how k is related to the various species of A in the column.

$$k = \frac{m_{AL}}{m_A + m_{AS}} \tag{A.1}$$

$$k = \frac{[A\text{–}L]}{[A] + [A\text{–}S]} \tag{A.2}$$

In Eq. (A.1), the terms m_{AL}, m_A and m_{AS} refer to the moles of A–L, A and A–S at equilibrium. In Eq. (A.2), [A–L] is the apparent concentration of the analyte–ligand complex in the column (i.e., m_{AL}/V_M, where V_M is the column void volume) and [A] or [A–S] are the corresponding concentrations of the analyte and analyte–ligand complex in the mobile phase.

Based on the reactions given in Eqs. (1) and (2), the following equilibrium expressions can be written for the formation of A–L and A–S.

$$K_{AL} = \frac{[A\text{–}L]}{[A][L]} \tag{A.3}$$

$$K_{AS} = \frac{[A\text{–}S]}{[A][S]} \tag{A.4}$$

In this model, the mass balance equation for S is given by Eq. (A.5), where C_S is the total or analytical concentration of this agent.

$$C_S = [S] + [A\text{–}S] \tag{A.5}$$

This expression can be rewritten in the form shown below if Eq. (A.4) is rearranged in terms of [A–S] and substituted into Eq. (A.5).

$$C_S = [S] + [S]K_{AS}[A] \tag{A.6}$$

From Eq. (A.6), it can be seen that the total concentration of solubilizing agent, C_S, may be used in place of the actual free concentration of this agent, [S], if the value of [A] is sufficiently small to make the term K_{AS} [A] much less than one. Thus, this is one of the requirements that must be met when selecting the amounts of injected analyte to obtain linear elution conditions.

If Eqs. (A.3) and (A.4) are substituted into Eq. (A.2), this gives the following expression.

$$k = \frac{K_{AL}[A][L]}{[A] + K_{AS}[A][S]} \tag{A.7}$$

If the reciprocal of this equation is taken, a linear relationship is then obtained between $1/k$ and [S], as given in Eq. (3). As discussed earlier, this provides a means for measuring K_{AS}.

Appendix B. Derivation of Eq. (4) for analytes interacting with multiple immobilized ligands

If analyte A binds to a series of independent immobilized ligands or ligand sites L_1, L_2, ..., L_m, the equilibrium expressions for these interactions will be given by Eqs. (A.8)–(A.10).

$$K_{AL1} = \frac{[A–L_1]}{[A][L_1]} \tag{A.8}$$

$$K_{AL2} = \frac{[A–L_2]}{[A][L_2]} \tag{A.9}$$

$$K_{ALm} = \frac{[A–L_m]}{[A][L_m]} \tag{A.10}$$

For this system, the total amount of all analyte–ligand complexes is given by Eq. (A.11).

$$[A–L]_{tot} = [A–L_1] + [A–L_2] + \cdots + [A–L_n] \tag{A.11}$$

If Eqs. (A.8)–(A.10) are rearranged to solve for each analyte–ligand concentration and the resulting expressions are substituted into Eq. (A.11), this gives the result shown in Eq. (A.12).

$$[A–L]_{tot} = K_{AL1}[A][L_1] + K_{AL2}[A][L_2] + \cdots + K_{ALn}[A][L_m] \tag{A.12}$$

If Eq. (A.12) is used to substitute $[A–L]_{tot}$ in place of [A–L] in Eq. (A.2), this produces the following expression.

$$k = \frac{\sum_{i=1}^{m} K_{ALi}[L_i]}{1 + K_{AS}[S]} \tag{A.13}$$

By taking the reciprocal of this relationship, the final result shown in Eq. (4) is obtained.

Appendix C. Derivation of Eq. (5) for analytes interacting with multiple soluble ligands

If a total of "n" moles of S can bind per mole of A to form a 1:n complex, the overall formation constant (K_{ASn}) for the production of the complex $A–S_n$ will be given by Eq. (A.14).

$$K_{ASn} = \frac{[A–S_n]}{[A][S]^n} \tag{A.14}$$

Under these conditions, the retention factor for A is now described by Eq. (A.15).

$$k = \frac{[A–L]}{[A] + [A–S_n]} \tag{A.15}$$

By substituting Eqs. (A.11) and (A.14) into Eq. (A.15), the following expression is created.

$$k = \frac{\sum_{i=1}^{m} K_{ALi}[L_i]}{1 + K_{ASn}[S]^n} \tag{A.16}$$

If the reciprocal of both sides is taken in Eq. (A.16), this leads to the relationship shown in Eq. (5).

Appendix D. Derivation of Eq. (6) for analytes with secondary interactions

For a system that has independent sites involved in specific binding and secondary interactions, the total retention factor for A (k_{tot}) will be given by the expressions shown in Eqs. (A.17) and (A.18).

$$k_{tot} = \frac{\sum_{i=1}^{m} m_{ALi} + m_{AL'}}{m_A + m_{AS}} \tag{A.17}$$

$$k_{tot} = \frac{\sum_{i=1}^{m} m_{ALi}}{m_A + m_{AS}} + \frac{m_{AL'}}{m_A + m_{AS}} \tag{A.18}$$

In these relationships, m_{ALi} is the moles of A that is bound at equilibrium with specific ligand site L_i and $m_{AL'}$ is the moles of A bound to the secondary sites. Other terms in this equation are the same as defined previously.

If the retention factor due to binding of A with the immobilized ligand is referred to k_L and the retention factor due to secondary interactions is k_s, then Eq. (A.18) can be rewritten as follows.

$$k_{tot} = k_L + k_s \tag{A.19}$$

$$\frac{1}{k_{tot} - k_s} = \frac{1}{k_L} \tag{A.20}$$

The final result shown in Eq. (6) is then obtained by simply combining Eq. (A.20) with Eq. (5).

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