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Guidelines in selecting ligand concentrations for the determination of binding constants by affinity capillary electrophoresis

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Abstract

This study examined various factors that affect the selection of ligand concentrations when using affinity capillary electrophoresis (ACE) for the determination of equilibrium constants in free solution. Two groups of model systems were used in this work: the binding of nitrophenols to α- or β-cyclodextrin and the binding of D- or L-tryptophan to human serum albumin (HSA). Both systems gave 1:1 binding behavior in the ACE studies and good fits to previous equations derived to describe the shift in analyte mobility that occurs as the ligand concentration of the running buffer is varied. Some practical factors limiting the range of ligand levels that could be used in such studies included the relative amount of injected analyte, ligand solubility and the ligand's background signal. More fundamental factors included the size of the equilibrium constant for the system being investigated, the relative range of mobilities over which the analyte peak might be observed, the precision of the mobility measurements and the number of analytes present in the sample. Equations and graphs were developed for illustrating each of these latter items and their role in determining the range of ligand concentrations that could be used in ACE binding constant measurements. The results predicted by these equations and graphs showed good agreement with those observed experimentally, and should prove useful in optimizing ACE conditions for other solutes and ligands. ©1997 Elsevier Science B.V.

Keywords: Equilibrium constants; Binding constants; Ligand concentrations; Nitrophenols; Tryptophan; Cyclodextrins; Human serum albumin

1. Introduction

Electrophoresis has long been recognized as a useful tool for examining the nature and strength of biological interactions (see Refs. [1–3] for reviews and Refs. [4,5] for additional examples). Although many different strategies can be used to examine solute–ligand binding by this method, these approaches are generally based on the change in analyte mobility that occurs as this species binds to a ligand present in the electrophoretic system. With the continuing development of capillary electrophoresis (CE), there has been increasing interest in the use of CE for these types of studies. This has given rise to a method now known as affinity capillary electrophoresis (ACE) [6,7]. ACE has already been used to measure binding constants in a number of systems, including the interactions of lectins with sugars [8–10], albumin with drugs [11,12], vancomycin with peptides [7,13–17], enantiomers with cyclodextrins [7,18–20], carbonic anhydrase with drugs or cations [16,21–25] and antibodies with antigens [26,27], in addition to several other examples [24,25,28–33]. Advantages of ACE *versus* traditional electrophoresis include its speed, resolving power and ability to work with small amounts of ligand or analyte.

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If an analyte and ligand have relatively fast association and dissociation, one approach that can be used to study their interactions is to directly inject the analyte into a CE running buffer containing a soluble ligand (e.g., see Fig. 1). In this method, the shift in the apparent mobility of the analyte is examined as the ligand concentration in the running buffer is varied [8–30]. The observed relationship between mobility and ligand concentration is then used to determine the binding constants for the system. One benefit of this approach is that it directly examines the interactions of the analyte and ligand in solution. Since the CE system also acts to separate the analyte from other sample components, this method can often be used with impure samples or it can be used to simultaneously study the binding of several different compounds with the ligand of interest [9,12,14,15,18–21].

Although the use of ACE for binding constant measurements has many potential advantages, there is still relatively little information regarding the experimental conditions that are most appropriate for this technique. For example, it is known that the proper choice of ligand concentration is important in ACE [18,22,34–36], but there has been little work examining those factors which determine the range of ligand levels that should be employed in ACE binding constant determinations. This report will examine such factors through the use of several model solute–ligand systems, including the binding of nitrophenols to cyclodextrins and the interactions of D- and L-tryptophan with the protein human serum albumin (HSA). These systems were chosen as models since: (1) they have known stoichiometries and equilibrium constants for their binding [34,37–41]; (2) they have relatively fast association and dissociation processes [37,38,42], thus making them amenable to direct solution-phase studies in CE; and (3) they represent two different classes of solute–ligand systems (i.e., the interaction of a solute with a relatively small ligand vs. solute binding with a biomacromolecule). Factors to be examined with these systems will include the roles that analyte concentration, binding affinity, degree of peak separation and peak precision play in determining the range of ligand concentrations that can be used for binding constant measurements in ACE. From the observations made with these models, general guidelines and optimization tools will be developed that can be applied to other solute–ligand systems.

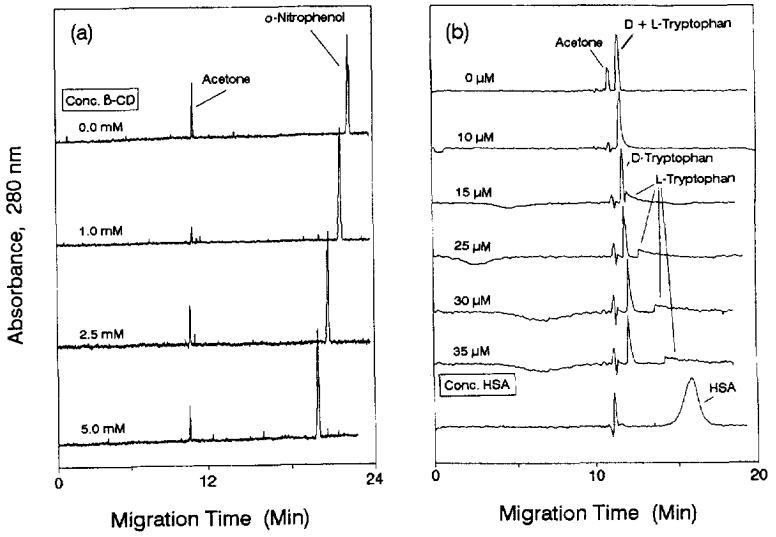

Fig. 1. Injection of (a) *o*-nitrophenol in the presence of various concentrations of β-cyclodextrin (β-CD) in the running buffer and (b) D- and L-tryptophan in the presence of various concentrations of HSA. The samples contained 100 μ*M* *o*-nitrophenol or 50 μ*M* D/L-tryptophan and 0.5% (v/v) acetone. In (a) the applied voltage was 20 kV and the total capillary length was 100 cm; in (b) the applied voltage was 18 kV and the total capillary length was 50 cm. All other conditions were the same as described in Section 2.

1.1. Theory

The 1:1 binding of an analyte (A) to a ligand (L) in solution to form an analyte–ligand complex (A–L) can be described by the following reaction and association equilibrium constant (K_a):

$$A + L \overset{K_a}{\rightleftharpoons} A - L \quad (1)$$

$$K_a = \frac{[A - L]}{[A]\,[L]} \quad (2)$$

where [] represents the molar concentration of each species in solution. In CE, if the injection of A into a capillary that contains a fixed concentration of L produces an analyte–ligand complex that has a different electrophoretic mobility than that seen for A alone (and the association plus dissociation of A with L is relatively fast), then the binding of A with L should result in a shift in the apparent migration time or velocity of A as the ligand concentration is varied. The degree of this shift can be described through the use of a relative mobility index (k''), as shown below [8,9,34]:

$$k'' = (\mu_r - \mu_A)/(\mu_{AL} - \mu_A) \quad (3)$$

where μ_r is the observed net mobility for A when injected in the presence of a given concentration of L, μ_A is the net mobility for A in the absence of L, and μ_{AL} is the net mobility for the pure analyte–ligand complex (e.g., A in the presence of a large excess of L). According to Eq. (3), k'' should have a value between one and zero (i.e., an observed migration time or velocity for A that occurs at or between the values expected for the pure analyte and the fully bound analyte–ligand complex) [34]. Although comparable ratios based on electrophoretic mobilities, velocities or migration times have also been used in ACE [7,12,14,15,18–23], the k'' index given in Eq. (3) was chosen for this work, since it is particularly convenient in illustrating how ligand concentrations might be selected for binding constant measurements (e.g., see Eq. (8) and Fig. 4 in Section 3).

One common difficulty when using ACE for binding studies is that the addition of ligand to the running buffer can change the viscosity of this solvent, which in turn causes the values of μ_A and μ_{AL} (as well as μ_r) to vary with ligand concentration [8,18,23,43]. This is undesirable since it means that k'' will be affected by the ligand through more than one mechanism, thus hindering the use of this parameter for binding constant measurements. To overcome viscosity-induced shifts in mobilities, one method that can be employed is to use mobility ratios in the calculation of k'':

$$k'' = (M_r - M_A)/(M_{AL} - M_A) \quad (4)$$

In Eq. (4), the mobility ratios M_r, M_A and M_{AL} are given by relationships $M_r = \mu_r/\mu_{eo}$, $M_A = \mu_A/\mu_{eo}$, and $M_{AL} = \mu_{AL}/\mu_{eo}$, where μ_{eo} is the mobility due to electroosmotic flow observed at each given ligand concentration for a non-reactive neutral marker [43]. Eqs. (3,4) are mathematically equivalent, since Eq. (4) is obtained by simply dividing both the numerator and denominator of Eq. (3) by μ_{eo}; however, Eq. (4) has a major advantage when used in binding studies since mobility ratios have been shown to be independent of the changes in solvent viscosity that can occur when ligands like cyclodextrins or proteins are added to CE running buffers [43]. This is in contrast to the behavior of ordinary electrophoretic mobilities, which can exhibit significant shifts due to ligand-induced changes in viscosity [8,18,23,43].

In order to use k'' to examine solute–ligand binding, it is necessary to relate this to the equilibrium constant for the system of interest. This can be done by using the following relationship between k'' and K_a for a solute–ligand system with a 1:1 interaction [8,9,34]:

$$k'' = K_a\,[L]/(1 + K_a\,[L]) \quad (5)$$

By taking the inverse of both sides of Eq. (5), the following alternative expression can be obtained [8,9]:

$$1/k'' = 1/(K_a\,[L]) + 1 \quad (6)$$

Eq. (6) is useful for ACE binding studies since it predicts that a plot of $1/k''$ *versus* $1/[L]$ for a system with 1:1 binding will result in a linear relationship with a slope equal to $1/K_a$, thus providing the association equilibrium constant for the analyte and

ligand of interest. An alternative form of Eq. (6), shown here in terms of mobility ratios and with the numerator and denominator terms of k'' being separated, can also be used to determine K_a [9]:

$$\frac{1}{(M_r - M_A)} = \frac{1}{[(K_a[L])(M_{AL} - M_A)]} + \frac{1}{(M_{AL} - M_A)} \quad (7)$$

In the case of Eq. (7), a plot of $1/(M_r - M_A)$, or $1/(\mu_r - \mu_A)$ as used in Ref. [9], is made *versus* $1/[L]$ and K_a is determined from the ratio of the intercept to the slope. The advantage of using Eq. (6) is that the dependent variable ($1/k''$) is a unitless parameter, making this expression valuable in studying the optimization of ACE systems, as will be done in this work. The advantage of Eq. (7) is that it does not require any prior knowledge of M_{AL} (or μ_{AL}) for the calculation of k'', thus simplifying the overall experiments involved in K_a measurements.

One assumption made in Eqs. (5–7) is that the amount of injected analyte is small *versus* the local amount of ligand that it contacts in the capillary. In other words, it is assumed that linear isotherm conditions are present, or that the migration time measured for the analyte is independent of the analyte's initial sample concentration. A second assumption made in Eqs. (5–7) is that the analyte is only interacting with ligand in the running buffer and not with ligand adsorbed to the capillary wall [34]. Tests to determine whether or not such conditions are actually present for a given solute–ligand combination will be discussed later in this study.

2. Experimental

2.1. Reagents

The HSA (Cohn fraction V, essentially fatty acid free), D- and L-tryptophan and all cyclodextrins were purchased from Sigma (St. Louis, MO, USA); the nitrophenols were obtained from Aldrich (Milwaukee, WI, USA). Other chemicals used in this study were from Fisher Scientific (Fair Lawn, NJ, USA). All buffers and solutions were prepared with deionized water obtained from a Nanopure water system (Barnstead, Dubuque, IA, USA).

2.2. Apparatus

All experiments were performed with an ISCO Model 3850 capillary electrophoresis system (Lincoln, NE, USA), equipped with a circulating fan for temperature control. The CE capillaries used in the cyclodextrin studies were 50 or 100 cm (30 or 65 cm effective length, respectively) × 50 μm I.D. untreated fused-silica columns from Polymicro Technologies (Phoenix, AZ, USA); the capillaries used in the HSA experiments were 50 cm (30 cm effective length) × 50 μm I.D. CE 200–glycerol coated fused-silica columns from ISCO. Data were collected using a Chromlink or Thermochrom interface from LDC (Riviera Beach, FL, USA). The collected electropherograms were analyzed using programs written in Microsoft QuickBASIC (Redmond, WA, USA).

2.3. Methods

Samples were applied by using the vacuum injection mode supplied with the Model 3850 CE system. The typical injection time was 8 s, which corresponded to an injection volume of approximately 12 nl. The applied voltages in the cyclodextrin work ranged from 1 to 10 kV for the 50 cm uncoated silica capillary and from 2 to 20 kV for the 100 cm column (i.e., 20 to 200 V/cm). The applied voltage was 10 to 20 kV (i.e., 200 to 400 V/cm) for the experiments with HSA and D/L-tryptophan. The temperature of the CE system during each study was 25±0.4°C. The internal temperature of the CE capillary under each set of operating conditions was estimated using the electrophoretic mobility method of Burgi et al. [46] (Note: an alternative approach would be to use the procedure of Bello et al., as described in Ref. [47]).

The capillaries used in the nitrophenol–cyclodextrin studies were cleaned with 0.025 *M* sodium borate buffer (pH 10.0) before use with any other running buffers or buffer additives; the CE 200–glycerol coated capillaries were cleaned with 0.10 *M* sodium hydroxide and deionized water before use. All cyclodextrin and nitrophenol solutions were prepared in degassed 0.0125 *M* potassium phosphate buffer (pH 11.0). A 0.0125 *M* potassium phosphate running buffer, pH 7.4, was used in the tryptophan–HSA studies. The typical operating current obtained with the pH 11.0 buffer at 8 kV was 20 μA for a

50-cm column, and the typical current for the pH 7.4 buffer at 16 kV was 42 μA. The ligand solutions prepared in each buffer were degassed and sonicated for at least 10 min prior to their application onto the CE system. At least ten different concentrations of cyclodextrin or HSA were used in each binding study. These concentrations ranged from 0.05 to 50 m*M* for the cyclodextrins and from 10 to 175 μ*M* for HSA.

The injected samples typically contained 10–100 μ*M* nitrophenol or 10–50 μ*M* D/L-tryptophan dissolved in the appropriate running buffer. Before injection, a small amount of acetone (0.5%, v/v) was added to each sample as a marker for electroosmotic flow. D/L-Tryptophan, acetone and the nitrophenols were detected by monitoring the absorbance at 280 nm. All samples were injected in duplicate or triplicate under each set of experimental conditions.

The migration time (t) of each peak was obtained by calculating the peak's first statistical moment [48]. The net electrophoretic mobility of the analyte (μ_{Net}) was determined from the analyte's migration time using the formula $\mu_{Net}=(L_{eff}\ L_{tot})/(tV)$, where L_{eff} is the effective capillary length from the injection end to the detector, L_{tot} is the total length of the capillary, and V is the applied voltage. The mobility ratio for the analyte was calculated from the experimentally measured mobilities, as discussed in Section 1.1, or by using the equivalent relationship $M=t_{eo}/t$, where t_{eo} is the migration time for a neutral marker (i.e., acetone) analyzed during the same run used to measure t for the analyte [43]. The values for k'' in Eq. (4) were usually calculated by estimating $(M_{AL}-M_A)$ from the intercept of plots made according to Eq. (7); however, in some cases, the value of $(M_{AL}-M_A)$ could be determined directly by measuring the difference in analyte mobility in the absence of ligand (M_A) and in the presence of a large excess of ligand (M_{AL}).

3. Results and discussion

3.1. General behavior of model systems

Some typical examples of electropherograms obtained with the model systems are shown in Fig. 1. These electropherograms are similar to those noted in other work using HSA and cyclodextrins as buffer additives (e.g., see Refs. [34,35,49]). As indicated by this figure, the net migration time for *o*-nitrophenol or D/L-tryptophan shifted as more ligand (i.e., β-cyclodextrin or HSA) was added to the running buffer. As higher concentrations of ligand were used, the extent of analyte–ligand binding increased and a larger shift in mobility was observed. Also note that those analytes with the greatest affinity for the ligand (e.g., L-tryptophan vs. D-tryptophan) exhibited the largest change in mobility when working at low ligand concentrations. It is these shifts in mobility and their relation to binding affinity that make such studies useful in the determination of solute–ligand equilibrium constants.

As mentioned earlier, the use of Eqs. (5–7) for accurate equilibrium constant measurements requires that there be little or no active ligand adsorbed within the CE system. No observable adsorption occurs with α- or β-cyclodextrin under the conditions used in this study, but adsorption can be an important factor when the ligand is a protein like HSA [12,32,34,44,45]. To avoid adsorption of proteins, it is necessary to carefully select the ionic strength and pH of the running buffer or to use capillaries that have been treated to minimize their protein interactions [12,32,44,45]. In this case, the latter method was chosen since it was desired to keep the pH and ionic strength of the running buffer as close as possible to those used in previous reports (e.g., see Ref. [34] and references therein). Under the given buffer conditions (i.e., a neutral pH and low-to-moderate ionic strength) it was found in earlier, frontal analysis experiments that approximately 0.7 monolayers of HSA can adsorb to the wall of an uncoated CE capillary [34]. When the same experiment was performed with HSA in the presence of the CE 200–glycerol coated capillary used here, no measurable amounts of adsorbed HSA were detected. Furthermore, studies in which small amounts of HSA were repeatedly injected onto the coated capillary gave consistent migration times and peak areas (i.e., only ±4.5% variation in the mobility ratio for HSA vs. a neutral marker and ±5% variation in the HSA peak area over a series of five sequential injections). All of this data indicates that HSA adsorption to the coated capillary was not a major problem in this work.

Throughout this study there was a noticeable change in the mobility of the neutral marker (i.e., a

measure of electroosmotic flow) as the concentration of HSA or cyclodextrin was varied; this was presumably due to changes in the solvent's viscosity as a result of adding ligand to the running buffer, as discussed in Section 1.1. For example, the addition of 15 m*M* α-cyclodextrin to the running buffer caused the mobility (and migration time) for the neutral marker to change by 12%. In the same manner, the addition of 50 μ*M* HSA caused the neutral marker's mobility to shift by 13%. Similar shifts were observed for charged solutes that had little or no interaction with the ligand being tested (e.g., pyronine Y for α-cyclodextrin or cytochrome *c* for HSA); this indicated that such shifts affected all injected solutes. However, when the mobilities for the charged, non-interacting solutes were normalized vs. those measured for the neutral marker, the resulting mobility ratios showed only random variations of ±1.0% or less. This agrees with previous theoretical work which demonstrated that the use of such ratios could cancel out any effects on the mobilities that may be caused by changes in the viscosity of the running buffer [43]. Because of these theoretical and experimental results, mobility ratios were used in all later experiments as the preferred means for describing analyte mobility during ACE binding studies.

Another requirement of using Eqs. (5–7) in binding constant measurements is that the migration of the analyte in the CE system (i.e., its mobility, velocity or migration time) must be independent of the analyte's initial concentration [22]. This requires that the amount of injected analyte be small *versus* the local amount of ligand in the CE system. This was tested by varying the concentration of each analyte at a given ligand level and determining the mobility ratio for the analyte under each set of conditions. Fig. 2a shows data obtained during such a study for *p*-nitrophenol and α-cyclodextrin. The greatest change in mobilities occurred at high sample concentrations and low ligand levels. Either of these situations produces a case in which the amount of injected analyte approaches the amount of ligand in the CE capillary, thus resulting in non-linear isotherm conditions. Similar curves were obtained for the other solute–ligand systems examined throughout this work. From these plots, it was possible to determine what concentrations of injected analyte

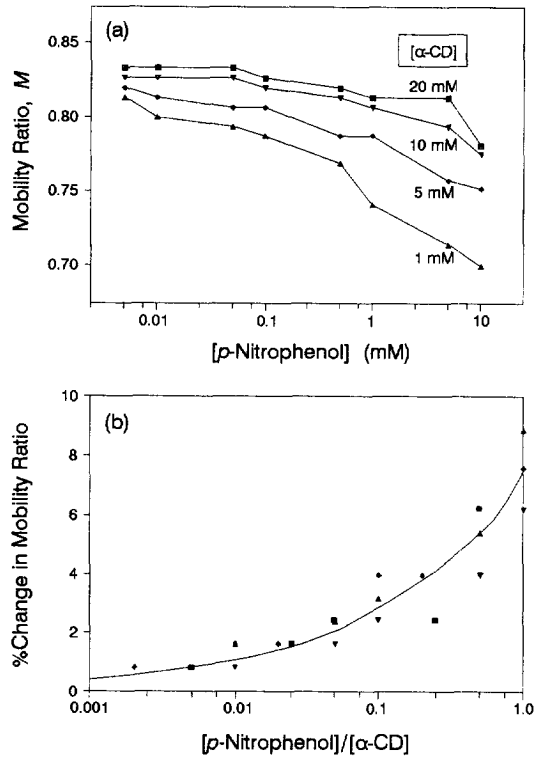

Fig. 2. Effect of analyte concentration on the apparent mobility ratio (*M*) measured for *p*-nitrophenol at various running buffer concentrations of α-cyclodextrin (α-CD). The top graph (a) shows the actual mobility ratios determined at each sample concentration. The bottom graph (b) shows the relative change in the *M* (vs. the value measured for the lowest concentration sample) as a function of the ratio of the *p*-nitrophenol and α-cyclodextrin concentrations. The α-cyclodextrin concentrations used in both the top and bottom graphs were 1 m*M* (▲), 5 m*M* (♦), 10 m*M* (▼) and 20 m*M* (■). The applied voltage was 20 kV and the total capillary length was 100 cm. All other conditions were the same as described in the text.

could be used without producing large changes in the apparent mobilities. For example, all later experiments in this study were done at analyte levels that gave a sample-size dependent change of 1–3% or less in the apparent mobility ratios (i.e., a level approximately equal to the typical variation in the mobility ratio measurements).

After appropriate experimental conditions had been established for each ACE model system, the migration of the test solutes was analyzed at several different ligand concentrations. Fig. 3 gives examples of typical graphs obtained with these models

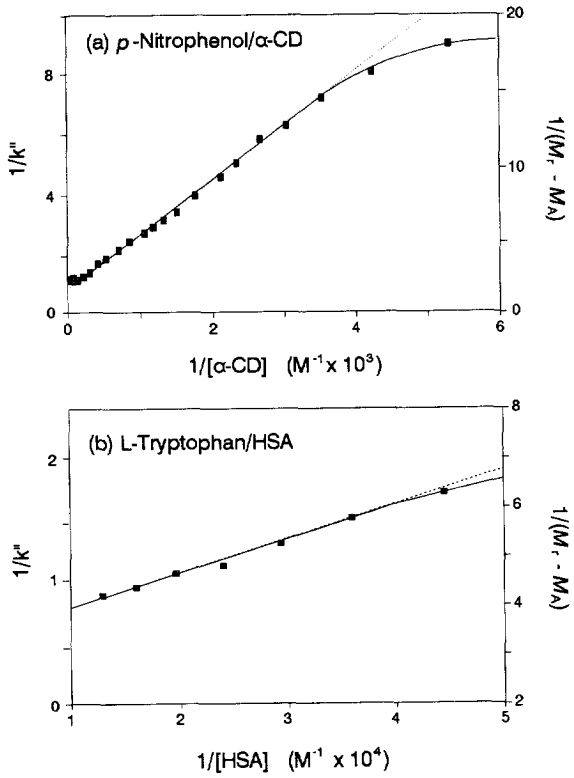

Fig. 3. Change in the mobility of (a) *p*-nitrophenol as a function of α-cyclodextrin (α-CD) concentration in the running buffer and of (b) L-tryptophan at various concentrations of HSA. The left-hand and right-hand sides of the graphs show the results plotted according to Eq. (6) and Eq. (7), respectively. In (a) the applied voltage was 20 kV, the total capillary length was 100 cm, and the sample concentration was 100 μ*M*. In (b) the applied voltage was 18 kV, the total capillary length was 50 cm and the sample concentration was 50 μ*M*. All other conditions were the same as given in the text. The dashed lines in both graphs show the best-fit linear response.

when the ACE data were plotted according to Eqs. (6,7). For each model system examined in this work, such plots gave linear behavior over a fairly broad range of ligand concentrations, as predicted by Eqs. (6,7) for solute–ligand systems with 1:1 interactions. It should be noted that some graphs (e.g., Fig. 3a) did show deviations from linearity at extreme ligand levels; some reasons for these deviations will be discussed in Section 3.2. Overall, the correlation coefficients obtained in the linear region of these graphs (i.e., the range of results within ±5% of the best-fit line) were between 0.9099 and 0.9976 (mean, $r=0.9781$) for the nitrophenol–cyclodextrin systems and between 0.9677 and 0.9946 (mean, $r=0.9812$) for D/L-tryptophan and HSA. The number of data points included in this linear region varied from 4–17 for the nitrophenol–cyclodextrin systems (mean, $n=10$), and from 7–8 for D- and L-tryptophan plus HSA. In addition, the K_a values measured from these plots showed good agreement with previous literature results [34,39–41] obtained when using comparable buffers and temperatures (i.e., an estimated internal capillary temperature of 26°C for the nitrophenol–cyclodextrin K_a studies and 37°C for the tryptophan–HSA work). From these results, it was concluded that the nitrophenol–cyclodextrin and tryptophan–HSA systems were good models of 1:1 binding behavior under the given CE operating conditions.

3.2. Selection of ligand concentrations for binding studies

The data obtained with the nitrophenol–cyclodextrin and tryptophan–HSA systems helped identify several potential factors to consider in the selection of ligand concentrations for equilibrium constant measurements by ACE. One important factor was the concentration of analyte used. At one extreme, a large enough analyte concentration is needed so that this solute can be easily detected; this amount will vary from one analyte–ligand system to the next and will be determined by such things as the response of the analyte, the background signal due to other components in the sample or the running buffer, and the noise level of the CE detector. In contrast to this, it is also important to select an analyte concentration that is sufficiently small vs. the level of ligand, to avoid shifts in the apparent mobility, as discussed in Section 3.1. By more closely examining the data in Fig. 2a and related studies, it was possible to obtain some general guidelines in identifying how low the analyte concentration must be for ACE studies (see Fig. 2b). The apparent shift in the mobility ratio, *M*, for the analyte increased in a non-linear fashion as the ratio of the analyte vs. ligand concentrations increased. For *p*-nitrophenol and α-cyclodextrin, an analyte–ligand ratio of 1:100 gave a shift in *M* of only 1–2%, a 1:10 ratio gave a 2–4% variation and a 1:1 ratio gave roughly a 6–10% variation. The same

trends were observed for the other combinations of nitrophenols and cyclodextrins tested, as well as for the binding of D- or L-tryptophan with HSA. Based on these results, analyte–ligand concentration ratios of less than 1:10 were used in making initial estimates of the analyte concentrations that should be used in each system; this estimate was later confirmed by using plots like those in Fig. 2a. A similar approach should apply to other systems, but the exact levels of analyte that can be used in ACE experiments may vary depending on the volume of the injected sample and the strength or nature of the analyte–ligand interaction being studied.

Another practical consideration affecting the choice of ligand concentrations was the ligand's solubility in the running buffer. This was not a problem when working with HSA, where studies were done well below the solubility limit of this protein, but this factor did affect the range of ligand concentrations that could be used with the nitrophenol–cyclodextrin systems. In particular, this limited the ACE studies to β-cyclodextrin concentrations below 20 m*M* and α-cyclodextrin levels below 35 m*M*. Some signs that limited solubility were present include the presence of a turbid solution after adding ligand to the running buffer and a levelling off of plots like those in Fig. 3 at low values of 1/[L] (i.e., high ligand concentrations).

A response at the detector due to the ligand was a second practical item that affected the range of ligand concentrations amenable to ACE experiments. Since UV absorbance at 280 nm was used for detection in this work, no background signal was noted in the case of the cyclodextrins, which do not have any chromophores that absorb in this range. However, HSA does absorb at this wavelength [49] and caused a high background signal as more of this agent was added to the running buffer. With the particular CE system used in this study, the ACE experiments were limited to work at HSA concentrations below 180 μ*M* (12 g/l). When this limit was exceeded, a noisy baseline was produced, along with poorer limits of detection for the injected analytes.

Yet another factor to consider in the choice of ligand concentrations is the size of the association equilibrium constant for the system being examined. Eq. (6) was rearranged into the following form to examine the role that this factor plays in determining the appropriate ligand concentrations for ACE studies:

$$[L] = (1/K_a)(k''/\{1 - k''\}) \quad (8)$$

This new expression is useful in selecting ligand levels since it indicates what concentration of ligand ([L]) is required to achieve a given shift in analyte mobility (k'') as a function of the association equilibrium constant (K_a) for the analyte and ligand. Based on Eq. (8), universal plots showing the relationship between [L] and K_a at several values of k'' were prepared, as shown in Fig. 4. These plots show that

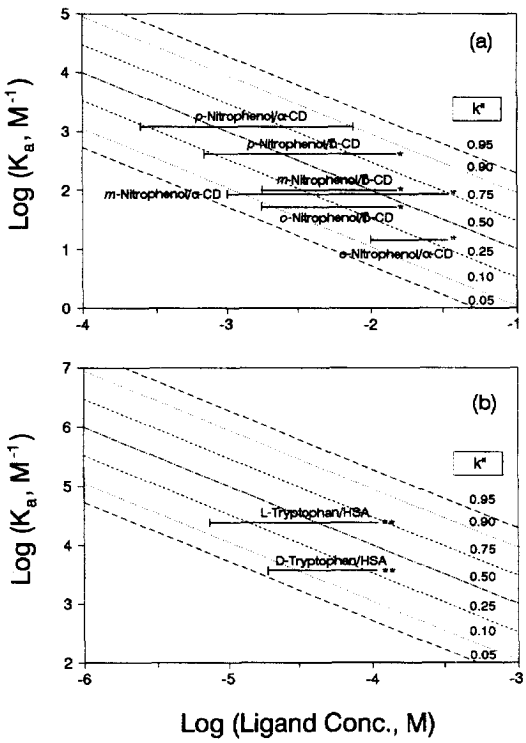

Fig. 4. Predicted and observed conditions for the measurement of association equilibrium constants (K_a) by ACE for the (a) nitrophenol–cyclodextrin and (b) tryptophan–HSA model systems. The dashed lines indicate the ligand concentrations needed to produce a given migration index (k'') at each K_a value. The solid, horizontal lines show the actual ranges of ligand concentrations that gave a linear response to Eqs. (6,7) for the systems examined in this study. The asterisks to the right of some horizontal lines indicate the points at which no further measurements could be performed due to problems with ligand solubility (*) or background signal arising from the ligand (**).

lower ligand concentrations must be used as the binding strength increases for the test system. This reflects the inverse relationship between [L] and K_a in Eq. (8), or the fact that strong analyte–ligand interactions will require smaller amounts of ligand to produce a situation in which the analyte is only partially complexed in solution. Note that the ideal situation for ACE binding studies occurs when the analyte peak appears exactly half way between the locations of the peaks for the non-complexed analyte and the fully bound analyte–ligand complex (i.e., $k''=0.5$). The ligand concentrations that produce this situation at various K_a values are given by the center diagonal line in Fig. 4. It can be seen from this plot and Eq. (8) that this line simply occurs at the point where $[L]=1/K_a$. This same condition has been noted previously by others to provide optimum resolution in related measurements and separations that use soluble ligands in CE running buffers [18,22,35].

Even though ligand concentrations that produce a k'' value of 0.5 are optimum for ACE studies, other conditions can be successfully used as well. For this reason, the ligand concentrations needed to produce k'' values of 0.05, 0.10, etc. are included in Fig. 4. The actual range of cyclodextrin and HSA concentrations observed to produce a linear response to Eqs. (6,7) are also shown. Under the conditions used in this work, the lowest k'' values that produced linear behavior occurred at, or around, 0.10, while the highest k'' values that gave a linear response extended up to about 0.90. According to Eq. (8), this represents at least an eighty-fold range in ligand concentration that is potentially suitable for ACE studies. In this study, it was not possible to sample this entire range for any one model system because of various practical considerations (Note: see asterisks in Fig. 4); this was generally caused by limited ligand solubility or ligand background signals, as discussed earlier. However, there were a few systems where these factors did not present a significant problem and that did show a fairly broad range of suitable ligand levels. For example, the binding of *p*-nitrophenol with both α- and β-cyclodextrin had a linear response that occurred over a 30-fold range in ligand concentration. A log plot of these concentrations gave a roughly symmetrical pattern about the point where $k''=0.5$, as predicted by Fig. 4. The interactions of *m*-nitrophenol with α-cyclodextrin covered a 35-fold range in ligand concentration and also showed a wide range of usable concentrations both above and below the optimum level at $k''=0.5$.

For those cases that did not suffer major problems due to ligand solubility or background signals, the main factor limiting the useful range of ligand concentrations was the resolution obtained between the peak for the partially complexed analyte and that for the free analyte or fully bound analyte–ligand complex. This item was capable of causing problems at either low or high ligand concentrations, and was found to be a function of the maximum range of analyte mobilities that could potentially be studied (i.e., the difference in peak position for the free analyte and pure analyte–ligand complex), as well as the precision of the mobility measurements. For example, if the ligand concentration was too low, then it was difficult to observe any shifts in the analyte's mobility vs. that for the free analyte (i.e., $M_r \approx M_A$). If the ligand concentration was too high, most of the analyte was bound and it was hard to distinguish the analyte's apparent mobility from that of the pure analyte–ligand complex (i.e., $M_r \approx M_{AL}$). As a result, the levels of ligand producing only partial complexation of the analyte were again the most desirable for such work.

Fig. 5 illustrates the relationship between the range of k'' values over which usable shifts in analyte mobility could be measured as a function of the relative difference in the mobilities for the free analyte and analyte–ligand complex ($|M_{AL}-M_A|$), and the precision of the mobility determinations. This plot shows that as the maximum difference in relative mobility decreases (i.e., $|M_{AL}-M_A|$ becomes small), a greater precision is needed to determine the shifts in analyte mobility that occur at a given k'' value. For the experiments described in this study, the relative range in mobility ratios, $|M_{AL}-M_A|$, for the nitrophenol–cyclodextrin systems had an average value of 0.465 units (range, 0.447 to 0.486) and the typical precision of the mobility ratio measurements was 1–3%. Based on this information, the data in Fig. 5 predict that a maximum usable k'' range of approximately 0.10 to 0.90 would exist under these conditions, which is in excellent agreement with the experimentally observed ranges seen in Fig. 4a. For the tryptophan–HSA system, the mobility ratio range

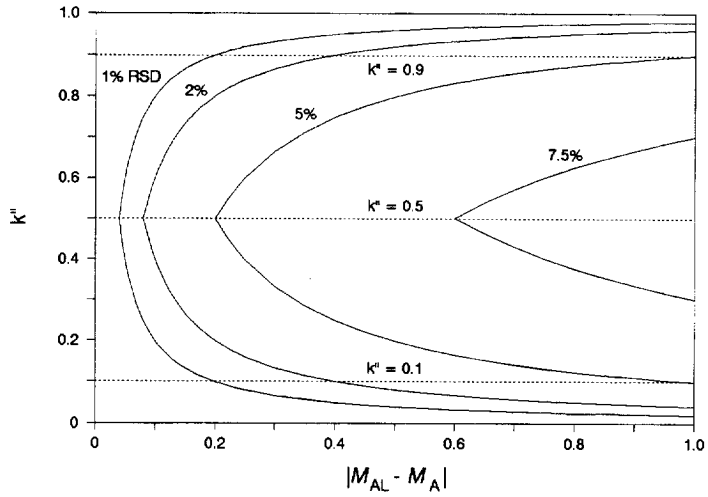

Fig. 5. Range of k'' values, at different levels of precision and maximum ranges of mobility shifts (the absolute value, $|M_{AL}-M_A|$), which give analyte peaks with mobility ratios that are statistically different from those for the free analyte (M_A) or the fully bound analyte–ligand complex (M_{AL}). The lines shown at each precision level represent the minimum or maximum k'' values needed to give a shift in peak position of 2 S.D. versus M_A or M_{AL}. The region to the right of each plot represents the range of usable k'' values at each value of $|M_{AL}-M_A|$; the regions to the left of these plots represent conditions where there is insufficient precision to discriminate between the analyte and reference peaks. For simplicity, it is assumed that the absolute precision in each plot is constant throughout the given range of mobility ratios.

was about 0.30–0.35 units and the precision was also 1–3%. This again gave a predicted lower end for the k'' range in Fig. 5 of around 0.10, which agrees with the lower k'' values seen experimentally in Fig. 4b (Note: recall that the upper end of the k'' range for D- and L-tryptophan was limited instead by the background signal due to HSA). Fig. 5 could similarly be used to help predict the usable k'' range for other solute–ligand systems.

When multiple analytes are being examined in a single run, another factor affecting the choice of ligand concentrations is the resolution between neighboring analyte peaks, which, in turn, depends on the width of these peaks and the differences in degree of ligand binding by each analyte. An example of this problem is given in Fig. 1b for mixtures of D- and L-tryptophan. The peak width in an ACE study will be determined by the efficiency of the CE system under the given operating conditions, but the degree of ligand binding will be determined by the association equilibrium constant for each analyte–ligand combination and the ligand's concentration. Some help in adjusting peak separation based on this second set of factors can be gained by going back to Eq. (8) and Fig. 4. For example, Fig. 4 predicts that D- and L-tryptophan will have significantly different k'' values only when working at HSA concentrations above 10^{-5} M (or 10 μM); this is in good agreement with the electropherograms shown in Fig. 1b. Similar agreement was noted when working with mixtures of *o*-, *m*- and *p*-nitrophenol and the cyclodextrin ligands. With both groups of model systems, the usable range of ligand concentrations was observed to be less for multianalyte samples than for single analyte preparations. Thus, although the simultaneous analysis of several solutes can help reduce the overall time required for ACE binding studies, greater care must be used in the selection of ligand concentrations for such experiments when performing K_a measurements.

4. Conclusions

This work used two model systems (i.e., the interactions of nitrophenols with α- or β-cyclodextrin and D- or L-tryptophan with HSA) to examine various factors that affect the selection of ligand

concentrations in ACE-based binding constant measurements. Some practical considerations that determined the range of usable ligand levels included the relative amount of injected analyte, ligand solubility (e.g., when working with α- or β-cyclodextrin), and the ligand's background signal (e.g., when working with HSA). Some general guidelines could be developed for one of these items (i.e., the maximum amount of analyte that could be used, as demonstrated in Fig. 2); however, the ligand solubility and background signal are inherent properties of the given system and must be considered on a case-by-case basis.

More fundamental factors that affected the choice of ligand concentrations included the size of the binding constant to be measured, the range of mobilities over which the analyte peak could be observed, the precision of the mobility measurements and the number of analytes present in the sample. These factors are important for all test systems and are governed by the nature of the ACE technique itself. Various equations and graphical methods were developed to illustrate the role of these factors in determining the appropriate concentrations for ACE studies. Examples included Eq. (8) and Fig. 4, which were used to show the relationship between binding affinity, ligand concentration and the relative shift in analyte mobility. Another example was Fig. 5, which indicated the effect of measurement precision on the usable range of conditions for binding constant determinations. The results obtained with these tools gave good agreement with those observed for the model experimental systems. From this work, it is expected that the same approaches should be useful as guides in optimizing ACE binding constant measurements for other solutes and ligands.

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