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Journal of Chromatography A, 721 (1996) 173–186

JOURNAL OF CHROMATOGRAPHY A

Examination of the origin, variation, and proper use of expressions for the estimation of association constants by capillary electrophoresis

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First received 15 February 1995; revised manuscript received 30 June 1995; accepted 13 July 1995

Abstract

Over the last several decades a variety of techniques have been developed to determine apparent equilibrium constants for molecular association in solution (e.g., to micelles, proteins, cyclodextrins, antibiotics, etc.). The relationships describing binding isotherms appear in several forms and have been given several different names. It is well known that most of these expressions are closely related and that some may be more advantageous than others for experimental or statistical reasons. In the case of electrophoresis, association constants are calculated from the relationship between ligand concentration and the measured electrophoretic mobility of the solute. This relationship has appeared in many forms that have been used numerous times at least since 1951. Recently they have reappeared in identical or slightly rearranged versions in several capillary electrophoresis (CE) studies. Some of these methods require the measurement of the electrophoretic mobility of the solute–ligand complex, a value that often cannot be accurately measured. Some systems require correction or normalization procedures in order to negate any changes in solute mobility that are not due to binding. The relationship between the various expressions that can be used to calculate binding constants with CE is shown. The advantages, limitations and proper use of the various approaches are discussed. Examples are given for both achiral and chiral analytes.

1. Introduction

Basic knowledge of equilibrium conditions and the association behavior of any dynamic chemical system is important if one is to evaluate and understand that system. Equilibrium constants for molecular association (also called binding constants or formation constants) have been measured using a variety of experimental approaches including spectroscopy [1–6], separations [7–20], calorimetry [21–24], potentiometry [25–28] and reaction kinetics [29–31]. For most of these techniques the system response is measured at varied ligand concentration and constant substrate concentrations and must differ for the bound and free analyte. The response can then be related to the relative concentrations of associated and free analyte, and to the binding constant and other physical properties inherent to the complex and free analyte (for example, extinction coefficients in spectrophotometry, chemical shifts in NMR, and mobilities in electrophoresis). All approaches can be related to each other mathematically and conceptually [32].

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0021-9673/96/$15.00
SSDI 0021-9673(95)00774-1

For electrophoresis, the equation which relates the apparent binding constant to the electrophoretic mobility of free and uncomplexed solute was presented for zone electrophoresis as early as 1951 by Alberty and King [12]. They derived an equation to describe the equilibrium between cadmium and iodide ions which considered multiple complexation steps. This equation related the measured mobility to the binding constants and the equilibrium concentrations of all species and was used in conjunction with moving boundary zone electrophoresis to calculate all of the binding constants. When reduced to a single binding scenario, this relationship is:

$$K[\mathrm{L}] = \left(\frac{\mu_f - \mu_i}{\mu_i - \mu_c}\right) \quad (1)$$

where K is the binding constant, [L] is the equilibrium concentration of uncomplexed ligand, and μ_f, μ_c are the electrophoretic mobilities of free and complexed solute; μ_i is the solute mobility measured at ligand concentration, [L]. This relationship has also been presented by numerous other workers for calculation of binding constants using electrophoresis [13–18]. Jokl used a semi-log plot of Eq. 1 to graphically estimate the apparent binding constants of ligands to metals using paper electrophoresis [13,33]. In addition, a relationship similar to Eq. 1, in terms of migration distances rather than mobilities, has been used to estimate dissociation constants using affinity electrophoresis where the ligand is immobilized in a gel [17,18,34,35]. Indeed, well over 100 publications in this area have appeared in the last 45 years using analogous or identical expressions as well as variations to account for effects of pH and other experimental parameters [12–17,33–38].

Recently this relationship has been re-introduced in various forms for capillary zone electrophoresis by Wren and Rowe for enantioselective complexation [20,39], and by Terabe and coworkers for micellar solubilization [40,41]. Subsequently, CE has been used by other workers for equilibrium constant determination using this same relationship or re-expressed versions of it [42–53]. The mobility of the complex, μ_c, must be measured in order to use Eq. 1 directly to estimate binding constants. This value can be measured in a few ways. A marker can be used which binds completely to the ligand, or the limiting electrophoretic mobility of the solutes can be measured at saturating ligand concentrations. Currently, well-defined markers are available only for micellar systems [40,41,54]. For many other systems, μ_c often cannot be accurately measured by the latter method due to experimental limitations such as ligand solubility [42] or capillary wall binding by the ligand [55]. Non-linear curve fitting methods have been used to fit experimental data to Eq. 1 in order to avoid these disadvantages [42–44].

In this work, Eq. 1 is related to the general form of the binding isotherm and several standard linear plotting methods are used to estimate apparent equilibrium constants. Binding constants are determined for both SDS and cyclodextrin systems for both chiral and achiral separations. The linear plotting methods do not require knowledge of the complex mobility, which can be difficult or impossible to measure. Also, precision and accuracy are known to improve when more than one data point is used. In this work, the methods will be outlined and compared for CE and their obvious relationship to other well known approaches for determining binding constants will be described.

2. Experimental

2.1. Materials

Sodium dihydrogen phosphate, sodium monohydrogen phosphate, sodium hydroxide, *p*-nitrophenol, *m*-nitrophenol, naphthalene, hydroquinone, 2-phenoxypropionic acid, 2-(4-chlorophenoxy)propionic acid, and mesityl oxide were purchased from Aldrich (St. Louis, MO, USA). Nylon filters, 0.45-μm, were purchased from Alltech (Deerfield, IL, USA), electrophoresis grade sodium dodecylsulfate (SDS) was from Bio-Rad (Richmond, CA, USA). Hydroxypropyl-β-cyclodextrin (average molar substitution =

0.6) and α-cyclodextrin was provided by Astec (Whippany, NJ, USA).

2.2. Methods

Capillary electrophoresis was performed using a P/ACE 2100 (Beckman, Palo Alto, CA, USA) thermostated at 20°C or 25°C. The unit was equipped with 37 cm × 50 μm (30 cm to detector) fused-silica capillary which was monitored at 280 nm. The chiral separations were performed with a Quanta 4000 (Waters, Millford, MA, USA) at ambient temperature (23°C) and solutes were detected at 214 nm. All samples were dissolved at less than 0.1 mg/ml in phosphate buffer–methanol (1:1) and injected using the pressure mode for 1 s. Run buffers were prepared by dissolving SDS or cyclodextrin in 50 m*M* sodium phosphate buffer. Phosphate buffers were prepared by dissolving the sodium hydrogen phosphate salts in water, adjusting the pH with sodium hydroxide and diluting to the final volume. All buffers and samples were filtered with 0.45-μm nylon filters.

The critical micelle concentration (CMC) of SDS was measured using a Surface Tensiometer 20 (Fisher, St. Louis, MO, USA) and found to be 0.4 m*M* in 50 m*M*, pH 7.0 phosphate buffer. This value is somewhat lower than that reported for SDS in 100 m*M* borate–50 m*M* phosphate buffer at pH 7.0 [56], but it is within the range of CMCs tabulated by Mukerjee and Mysels [57] for SDS in various electrolyte solutions. It is well known that salts reduce the CMC of ionic surfactants and that the CMC depression is dependent on the salt concentration and type. In addition, there have been many discrepancies in the CMC values reported by different laboratories under seemingly identical conditions [57]. These CMC measurements are extremely sensitive to the purity of the surfactant, salts and water used to prepare the solutions. For these reasons, the CMC was experimentally determined with the reagents used to prepare the CE solutions rather than using a previously reported CMC value which did not necessarily apply to this system.

Mesityl oxide was used as the electroosmotic flow (EOF) marker and quinine HCL was used to estimate the migration of the SDS micelle [54]. Migration times were corrected for changes in solution viscosity caused by changes in ligand concentration using the migration time of mesityl oxide [20,42]. Experimental data was manipulated and parameters were calculated using Least Squares software [58]. This program transforms the experimental data for graphing, performs weighted least squares linear regression, and performs propagation of error analysis for the slopes and intercepts. For the calculation of weighting factors it was assumed that the magnitude of the uncertainties in the measured migration data was the same for all data points. That is, it was assumed that the percent uncertainty in the experimentally measured data was not constant, with smaller values having greater relative error than larger values. In general, the weighting factors are directly proportional to the experimentally measured values raised to some power (depending on the data transformation). The weighting factors were then used in linear regression analysis. Weighted linear regression methods are discussed in detail in the Least Squares program [58] and by Connors [32]. Binding constants were determined from the slopes and intercepts according to the methods in Table 1. The software calculates the uncertainties in the slopes and intercepts using propagation of error analysis methods. For example, the errors in the slopes, m, were calculated from: $\delta m^2 = (\Sigma(\partial m/\partial y_i)^2\, \delta y_i^2$, where δm^2 is the square of the uncertainty in the slope. The operation $(\partial m/\partial y_i)^2$ is performed on the equation used to calculate the slope from the experimental data, and δy_i^2 is calculated from the actual y-values and those given by regression analysis. The slope and intercept uncertainties reported by the program represent the 67% confidence level with two deviations conforming roughly to the 95% confidence level [58]. The uncertainties in the binding constants and mobilities listed in Tables 2–5 were calculated by propagation of error methods using the errors in the slopes and intercepts given by the program. While weighted least squares methods were used to treat the data in this work, it should be noted that this is not

the main focus of this paper. In fact the conclusions would be largely unchanged if all plots were made by unweighted linear regression.

For all calculations, the equilibrium free ligand concentration was approximated as total concentration of ligand, i.e., $[L]_{free} = [L]_{total}$. This is a reasonable estimate when the total ligand concentration is much greater than the total solute concentration or the binding constants are not large [32]. This estimate cannot be used when a substantial amount of the ligand is complexed to the solute, that is, when K is large or when the solute concentration is significant compared to the ligand concentration. In these instances, $[L]_{free}$ is calculated as the difference between the total ligand concentration and the equilibrium bound ligand concentration: $[L]_{free} = [L]_{total} - [L]_{bound}$.

3. Results and discussion

It is well known that any 1:1 molecular association between substrate, S, and ligand, L:

$$S + L \Leftrightarrow S{:}L$$

can be described by the general form of the binding isotherm [32]:

$$y = \frac{dx}{f + ex} \tag{2}$$

The dependent variable, y, is the experimentally measured response of the system containing ligand and substrate. Typical experimental responses used include virtually any spectroscopic change (for example, change in the position or intensity of UV–Vis, fluorescence, NMR, IR, or circular dichroism signal) or a change in an analytical separation parameter (i.e., HPLC, TLC, GPC, electrophoretic mobility, etc.). x is the concentration of free ligand, and d, e, and f are constants or parameters related to the properties of the solute, ligand and complex. This general isotherm has been used to estimate binding constants using numerous analytical methods and these have been extensively reviewed by Connors [32]. Some notable examples of this equation are:

the fraction of weak acid, F_{HA}, in the acid form [32]:

$$F_{HA} = \frac{[H^+]}{K_a + [H^+]} \tag{3}$$

the spectrophotometric change in absorbance, ΔA, of 1:1 binding of substrate, S, with ligand, L [1–4]:

$$\frac{\Delta A}{b} = \frac{K_{11} S_t \Delta\varepsilon [L]}{1 + K_{11}[L]} \tag{4}$$

the Michaelis–Menten equation for enzyme kinetics which describes the initial reaction velocity, v, in terms of substrate concentration, [S] and maximum velocity, V_m [29–31]:

$$v = \frac{V_m[S]}{K_m + [S]} \tag{5}$$

and the measured change in the NMR chemical shift, Δ, with ligand concentration [5,6]:

$$\Delta = \frac{\Delta_{11} K_{11}[L]}{1 + K_{11}[L]} \tag{6}$$

Eq. 2 can be rearranged into many forms for graphical analysis including [32]:

$$\frac{1}{y} = \frac{f}{d}\frac{1}{x} + \frac{e}{d} \tag{7}$$

$$\frac{x}{y} = \frac{e}{d}x + \frac{f}{d} \tag{8}$$

and

$$\frac{y}{x} = -\frac{e}{f}y + \frac{d}{f} \tag{9}$$

These equations and their corresponding plots have been called the double reciprocal, y-reciprocal and x-reciprocal forms, respectively [32]. Historically they have been the preferred plotting methods for calculation of binding constants and they have appeared in the literature with many names. The double-reciprocal plot is known as the Benesi–Hildebrand plot in spectrophotometry and the Lineweaver–Burk plot in enzyme studies, while the x-reciprocal plot is also called an Eadie plot in enzyme kinetics or a Scatchard plot in protein binding studies. While

Eqs. 7–9 appear to be identical (since one equation can be algebraically rearranged to produce the others) these plotting methods may not necessarily be equivalent due to the difference in uncertainties in the x and y variables before and after transformation for graphical analysis [32]. Rearranging an equation in order to make it easier to plot, to simplify experimental measurements, or to take advantage of the differences in the uncertainties in the x and y variables is a useful and legitimate endeavor. Of course, simply re-expressing an equation or derivation using different symbols in order to make it look original, is not. This has been discussed previously [59].

The equation for capillary electrophoresis which describes the relationship between the measured electrophoretic mobility of a solute in a solution containing a ligand is given by Eq. 1. This is the same equation, in terms of mobilities rather than migration times, first used by Terabe and co-workers to estimate capacity factors of solutes to micelles in CE [40,41]. Eq. 1 can be written in terms of capacity factors, k', rather than equilibrium constants through the following relationships:

$$k' = \frac{n_{A,micelle}}{n_{A,aqueous}} = \frac{[A]_{micelle}}{[A]_{free}[Micelle]_{free}} \times [Micelle]_{free} = K[L]$$

where $n_{A,micelle}$ and $n_{A,aqueous}$ are the number of moles of solute in the micellar and aqueous phases, respectively. Capacity factors for solutes to micelles are usually calculated by measuring the mobilities (or migration times) of a solute in the presence and absence of micelles. There are several well characterized micelle markers (i.e., compounds assumed to be completely associated with the micelles) that are used to measure the mobility of the micelle [40,41,54]. The capacity factors are then calculated from this data at a single micelle concentration. Binding constants are usually estimated graphically using Eq. 1 for other systems where markers are not available. The procedure requires measuring the mobility of a solute at varying ligand concentrations, measuring the limiting mobility of the solute at saturating ligand concentrations, extrapolating the mobilities to a zero ligand concentration reference state, and plotting the ratio of the mobility differences versus the free ligand concentration [20,39]. This method has several drawbacks. The graph should be forced through the origin since the presence of an intercept does not have physical meaning. In addition, the electrophoretic mobility of the solute–ligand complex (μ_c) can be difficult or even impossible to determine for most systems [42]. High ligand concentrations are often required and extrapolation of data back to a reference point of zero ligand concentration can introduce error. In addition, the equilibrium constant can be very sensitive to the value of μ_c, depending on the relative magnitudes of μ_c and μ_i. Other workers have fit experimental data to Eq. 1 or other forms of Eq. 1 using non-linear curve fitting methods in order to avoid these problems [42–44].

When using CE, the ideal data analysis methods would not require independent knowledge of μ_c, but would allow the comparison of results and verification of the binding scenario using different approaches. This is done by solving Eq. 1 for the experimentally measured variable, μ_i, to give:

$$\mu_i = \frac{\mu_f + \mu_c K[L]}{1 + K[L]} \tag{10}$$

Eq. 10 can be transformed into the same mathematical form as the general binding isotherm, Eq. 2, by referencing all measured electrophoretic mobilities (i.e. μ_i and μ_c) to that of the free solute (μ_f) as shown below:

$$(\mu_i - \mu_f) = \frac{(\mu_c - \mu_f)K[L]}{1 + K[L]} \tag{11}$$

The various forms analogous to Eqs. 7–9 are:

$$\frac{1}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f)K}\frac{1}{[L]} + \frac{1}{(\mu_c - \mu_f)} \tag{12}$$

$$\frac{[L]}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f)}[L] + \frac{1}{(\mu_c - \mu_f)K} \tag{13}$$

and

$$\frac{(\mu_i - \mu_f)}{[L]} = -K(\mu_i - \mu_f) + K(\mu_c - \mu_f) \quad (14)$$

As with other instrumental techniques (vide supra) these forms are most convenient for graphical analysis. An equation similar to Eq. 14, a Scatchard-type equation, was introduced by Carpenter et al. for calculation of binding constants using affinity capillary electrophoresis [45] and subsequently used by others [46–49,52,60]. A linear, double reciprocal plotting method similar to Eq. 12 was presented by Hořejší in 1979 for evaluation of dissociation constants from traditional affinity electrophoresis data [61]. The relationship was originally derived by comparison to analogous expressions for affinity chromatography, and not from fundamental electrophoresis properties of the system. The equation was expressed in terms of migration distances rather than mobilities, and the ligand was immobilized so that $\mu_c = 0$. When these factors are considered in Eq. 12, it becomes equivalent to that used by Hořejší. Recently, Kuhn et al. derived and used Eq. 12 to calculate binding constants for lectin–sugar systems [50]. Their relationship was derived from an equation analogous to Eq. 1. To our knowledge, Eq. 13 has not yet been used for estimating binding constants from CE data.

Table 1 lists the plotting forms and the methods of calculation for Eqs. 12–14. If the electroosmotic flow (EOF) velocity remains unchanged with changing ligand concentration, these equations can also be expressed in terms of migration times using the relationship [49]:

$$(\mu_i - \mu_f) = \frac{lL}{V}\left(\frac{1}{t_i} - \frac{1}{t_f}\right) \quad (15)$$

where L and l are the total capillary length and length to the detector, V is the run voltage, and t_i and t_f are the measured and free migration times of the analyte. If the EOF velocity changes with changing ligand concentration due to solution viscosity changes, the migration times should be referenced to zero ligand concentration using a neutral marker [20,42]. In the case of wall adsorption of the ligand, it has been shown that using a neutral ligand to correct for changes in the EOF is not a valid procedure [55]. In this instance, the electrophoretic mobilities (i.e., $\mu - \mu_{eof}$) should be calculated for the free solute and for the solute at each ligand concentration. From these values, $\mu_i - \mu_f$ can be calculated.

Table 1
Plotting forms of Eqs. 1, 12, 13 and 14

Equation/plotting method	K	$\mu_c - \mu_f$
1. Mobility ratio difference method		
$\frac{\mu_f - \mu_i}{\mu_i - \mu_c}$ vs [L]	slope	must be determined experimentally
12. Double reciprocal		
$\frac{1}{(\mu_i - \mu_f)}$ vs $\frac{1}{[L]}$	$\frac{\text{intercept}}{\text{slope}}$	$\frac{1}{\text{intercept}}$
13. y-Reciprocal		
$\frac{[L]}{(\mu_i - \mu_f)}$ vs [L]	$\frac{\text{slope}}{\text{intercept}}$	$\frac{1}{\text{slope}}$
14. x-Reciprocal		
$\frac{(\mu_i - \mu_f)}{[L]}$ vs $(\mu_i - \mu_f)$	−slope	$-\frac{\text{intercept}}{\text{slope}}$

In this work, the binding constants were measured for the association of naphthalene, *p*-nitroaniline, hydroquinone and *p*-nitrophenol to SDS, as well as those for *p*-nitrophenol and *m*-nitrophenol to α-cyclodextrin. The plots of solute mobility as a function of SDS concentration are shown in Fig. 1. Plots of Eqs. 12–14 are shown in Figs. 2–4 and Fig. 5 shows mobility ratio plot of Eq. 1 for the SDS systems. The concentration of SDS in the micelles was calculated by subtracting the CMC from the total SDS concentration. The same plots are shown in Figs. 6–10 for the binding of solutes to α-cyclodextrin. In addition, the applicability of these methods for the calculation of chiral selectivity is demonstrated for 2-phenoxypropionic acid and 2-(4-chlorophenoxy)propionic acid with hydroxypropyl-β-cyclodextrin. Plots showing the effect of hydroxypropyl-β-cyclodextrin concentration on solute mobility are shown in Fig. 11.

Tables 2 and 3 list the binding constants determined for different solutes to SDS and α-cyclodextrin by the various graphical techniques. The listed literature values were converted from

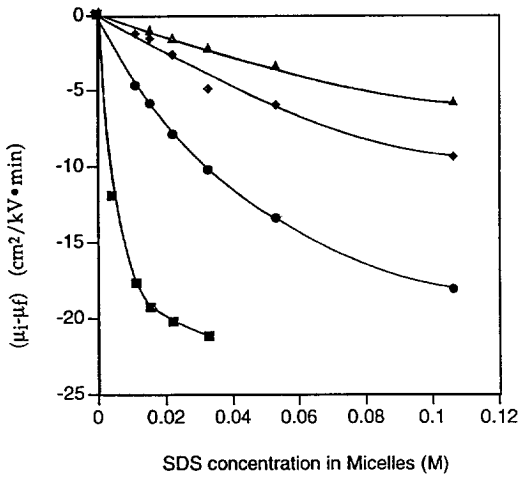

Fig. 1. Plots of the change in mobility of solutes as a function of SDS concentration in the micelles (i.e., C-CMC) at 20°C. These data points were used in conjunction with linear plots of Eqs. 12–14 for calculation of the apparent binding constants. The curves were drawn by hand to illustrate the general trends in the data. The solutes are: (■) naphthalene, (▲) hydroquinone, (●) *p*-nitroaniline, and (◆) *p*-nitrophenol. See Experimental for more details.

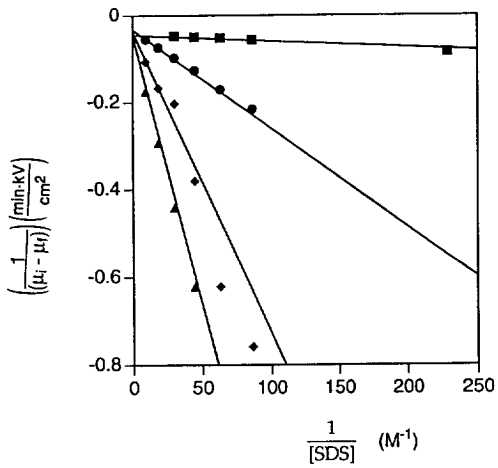

Fig. 2. Double reciprocal plot (Eq. 12) for SDS at 20°C with: (■) naphthalene, (▲) hydroquinone, (●) *p*-nitroaniline, and (◆) *p*-nitrophenol. [SDS] is the concentration of SDS in the micelles.

partition coefficients to equilibrium constants using the relationship: $K_{eq} = (K_p - 1)V$, where K_{eq} is the equilibrium constant, K_p is the partition coefficient and V is the molar volume of SDS (0.249 M^{-1}) [62].

Because the cyclodextrins are neutral ligands which cause significant changes in solution viscosity, the migration times were referenced to zero cyclodextrin concentration using a neutral

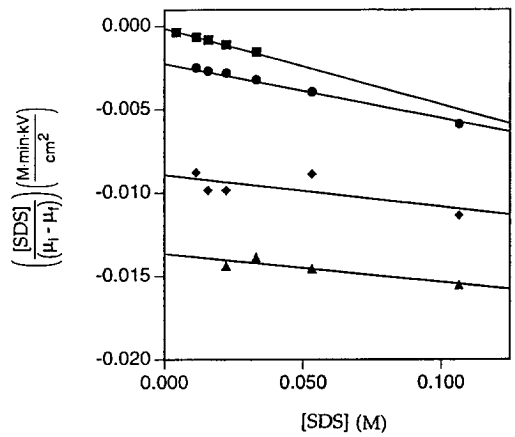

Fig. 3. *y*-Reciprocal plot (Eq. 13) for SDS at 20°C with: (■) naphthalene, (▲) hydroquinone, (●) *p*-nitroaniline, and (◆) *p*-nitrophenol. [SDS] is the concentration of SDS in the micelles.

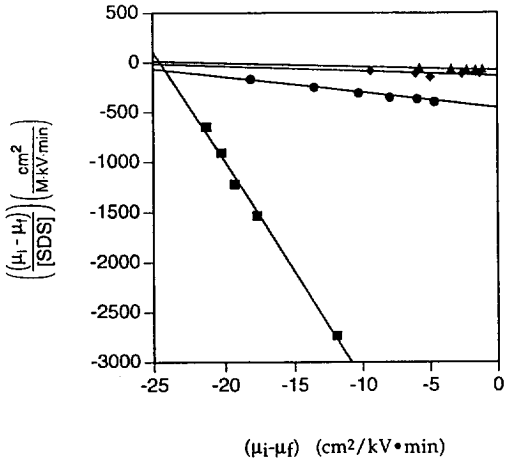

Fig. 4. x-Reciprocal plot (Eq. 14) for SDS at 20°C with: (■) naphthalene, (▲) hydroquinone, (●) p-nitroaniline, and (◆) p-nitrophenol. [SDS] is the concentration of SDS in the micelles.

marker. Binding constants to SDS were calculated with and without this correction for comparison. These values are listed in Table 2. The binding constants and the correlation coefficients for the SDS systems calculated from the corrected data did not differ significantly from those obtained without adjusting the raw data. Electrophoretic mobilities must be corrected when a solute or additive changes the electrophoretic mobility of the analyte due to factors other than

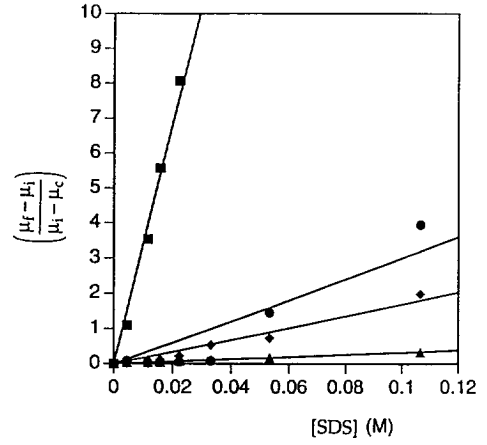

Fig. 5. Plot of Eq. 1 using experimentally determined complex mobilities for SDS at 20°C with: (■) naphthalene, (▲) hydroquinone, (●) p-nitroaniline, and (◆) p-nitrophenol. [SDS] is the concentration of SDS in the micelles.

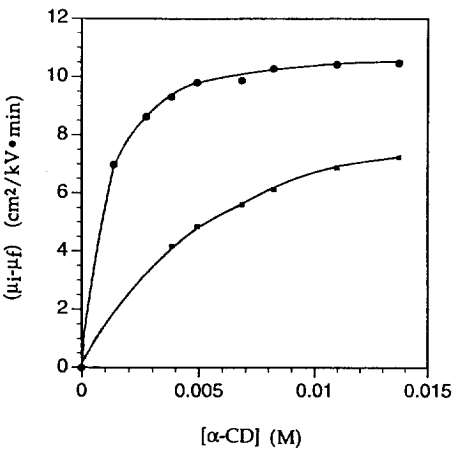

Fig. 6. Plots of the change in mobility of solutes as a function of α-cyclodextrin concentration at 25°C. These data points were used in conjunction with linear plots of Eqs. 12–14 for calculation of the apparent binding constants. The curves were drawn by hand to illustrate the general trends in the data. The solutes are: (■) m-nitrophenol and (●) p-nitrophenol.

molecular association. While the required correction for viscosity changes is well documented for neutral cyclodextrin ligands [20,42], charged species can change the electrophoretic mobilities of the solutes by changing the electric double layer, solution viscosity, or by capillary wall binding. Under these circumstances the correction methods needed to reference mobilities to zero ligand concentration may not be as direct.

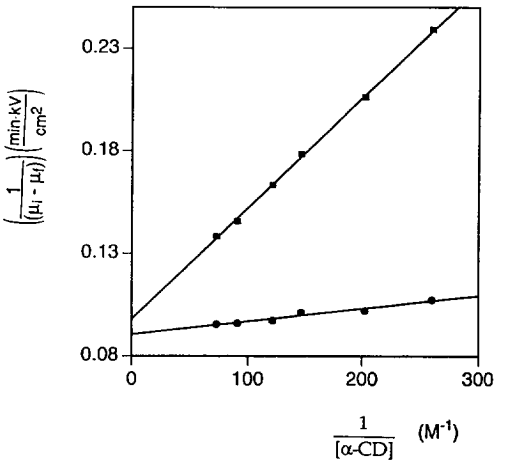

Fig. 7. Double reciprocal plot (Eq. 12) for α-cyclodextrin at 25°C with: (■) m-nitrophenol and (●) p-nitrophenol.

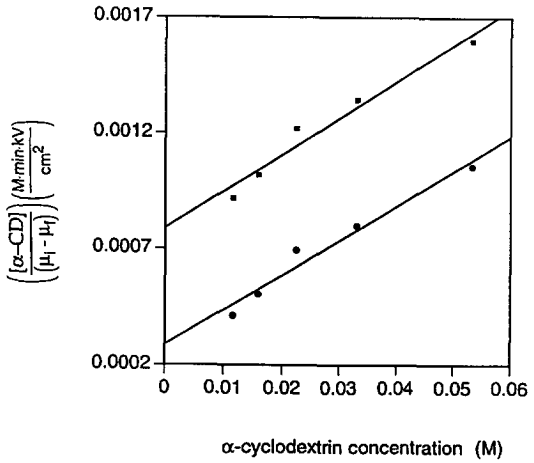

Fig. 8. y-Reciprocal plot (Eq. 13) for α-cyclodextrin at 25°C with: (■) m-nitrophenol and (●) p-nitrophenol.

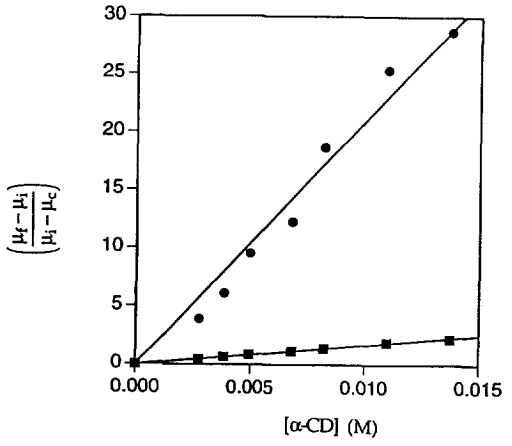

Fig. 10. Plot of Eq. 1 using experimentally determined complex mobilities for α-cyclodextrin at 25°C with: (■) m-nitrophenol and (●) p-nitrophenol.

The values of K obtained using Eqs. 12–14 are within the range of those reported using other techniques and the relative uncertainties are less than 10% in most cases. The plotting forms of Eqs. 12 and 13 (the double reciprocal and y-reciprocal plots) appear to be equivalent for this data when using weighted least squares analysis. This similarity has been reported for analysis of Michaelis–Menten parameters using corresponding plotting methods with weighted linear regression [63,64]. The relative merits of linear plotting methods for other types of experimental data have already been addressed in detail [32,63–65]. In general, it was found that linear plotting methods analogous to Eqs. 12–14 give parameters that are comparable to those obtained from non-linear curve fitting methods provided that the data is properly weighted for linear regression. Therefore, the choice of data analysis, either non-linear curve fitting methods

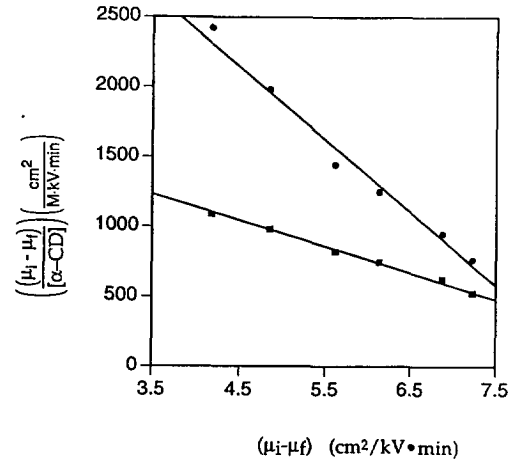

Fig. 9. x-Reciprocal plot (Eq. 14) for α-cyclodextrin at 25°C with: (■) m-nitrophenol and (●) p-nitrophenol.

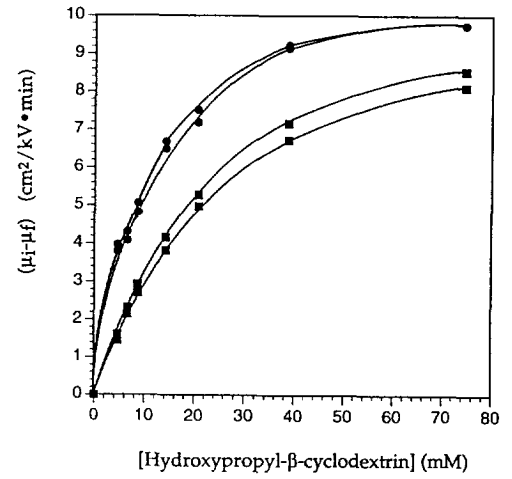

Fig. 11. Plots of the change in mobility of solutes as a function of hydroxypropyl-β-cyclodextrin concentration at 25°C. These data points were used in conjunction with linear plots of Eqs. 12–14 for calculation of the apparent binding constants. The curves were drawn by hand to illustrate the general trends in the data. The solutes are: (■) 2-phenoxypropionic acid enantiomers and (●) 2-(4-chlorophenoxy)propionic acid enantiomers.

Table 2
Binding constants for solutes to sodium dodecylsulfate in 50 mM, pH 7.0 phosphate buffer at 20°C

Compound	Binding constants (M^{-1})				
	Mobility ratio method	Double reciprocal	y-Reciprocal	x-Reciprocal	Literature values[a]
p-Nitrophenol					
not adjusted[b]	16.9 ± 1.4	9.0 ± 2.3	9.0 ± 2.3	5.9 ± 2.0	6.7[d], 7.9[e], 14[g], 25.7[h]
adjusted[c]	8.5 ± 2.5	5.3 ± 2.5	5.3 ± 2.5	5.1 ± 4.0	
p-Nitroaniline					
not adjusted[b]	26.0 ± 1.4	16.6 ± 0.3	16.6 ± 0.3	17.7 ± 0.3	21.4[d], 17.7[e]
adjusted[c]	30.0 ± 3.9	14.4 ± 0.8	14.4 ± 0.8	15.6 ± 1.2	26.9[f]
Hydroquinone					
not adjusted[b]	3.2 ± 0.1	4.3 ± 0.2	4.3 ± 0.2	4.4 ± 0.2	3.4[e]
adjusted[c]	3.5 ± 0.3	3.6 ± 0.4	3.6 ± 0.4	3.7 ± 0.4	
Naphthalene					
not adjusted[b]	349 ± 32	226 ± 15	226 ± 15	244 ± 14	78.2[e], 259[h], 242[j], 113–274[i]
adjusted[c]	428 ± 120	330 ± 52	330 ± 52	235 ± 42	

[a] Literature values were reported as partition coefficients (K_p) at 20°C. They were converted to equilibrium constants using the relationship [59]: $K_{eq} = (K_p - 1)V$, where V is the molar volume of SDS, 0.249 M^{-1}.
[b] Migration times were not adjusted to account for the change in the electroosmotic flow. Unadjusted electrophoretic mobilities were used driectly for plotting: $\mu_i = (lL/V)(1/t_i - 1/t_{EOF})$ where t_i is the measured migration time and t_{EOF} is the migration time of the neutral marker at a given SDS concentration.
[c] Migration times were adjusted to account for the change in the electroosmotic flow: $\mu_i^{adj} = (lL/V)(1/t_i^{adj-1}/t_{EOF}^0)$ where $t_i^{adj} = t_i(t_{EOF}^0/t_{iEOF})$; t_{EOF}^0 is the migration time of a neutral marker at zero ligand concentration.
[d] From Ref. [11].
[e] From Ref. [10].
[f] From Ref. [56] at 25°C.
[g] From Ref. [70].
[h] From Ref. [71].
[i] From Ref. [72].
[j] From Ref. [73].

Table 3
Binding constants for p-nitrophenol and m-nitrophenol to α-cyclodextrin in 50 mM, pH 11 phosphate buffer at 25°C

Compound	Binding constants[a] (M^{-1})				
	Mobility ratio method	Double reciprocal	y-Reciprocal	x-Reciprocal	Literature values[b]
p-Nitrophenol	2400 ± 300	1500 ± 200	1500 ± 200	1400 ± 300	1800 ± 300[c] 2290[d], 670[e]
m-Nitrophenol	440 ± 60	182 ± 5	182 ± 5	190 ± 10	202 ± 3[c]

[a] Migration times were adjusted for the change in the electroosmotic flow. See Table 2 for more details.
[b] Literature values were determined at 25°C in NaOH.
[c] Determined at pH 11.1 using a calorimetric method. From Ref. [65].
[d] Determined using UV spectroscopy. From Ref. [66].
[e] Determined at pH 10.0 using NMR. From Ref. [67].

or weighted linear plotting methods, can be left to the individual researcher. When the data is not weighted, the double reciprocal plot tends to place too much emphasis on the data points taken at the lowest ligand concentrations [32,63]. The Scatchard-type plot has been criticized for using the dependent variable on both the x and y axes, thus complicating statistical analysis of the data [64].

Table 4 compares the experimentally determined values of the complex mobilities, μ_c, to those determined using Eqs. 12–14. In the SDS system, μ_c is the mobility of the solute when it is incorporated within the micelle. This value is experimentally determined from the electrophoretic mobility of a micelle marker that binds completely to the micelle [40,41,54]. There are some differences between the μ_c values determined by the different methods. These differences may be due to the nature of the solutes and the micelle marker. Incorporation of the micelle marker or solutes into the micelle may change the electrophoretic mobility of the micelle. Solubilization of these compounds by the micelles can change the aggregation number of the micelles or it can alter the overall charge on the micelle if the solute or marker is charged. These factors can influence the electrophoretic mobility of the micelles by changing both their charge and size. For the SDS systems, μ_c values were determined from the mobility of a positively charged micelle marker, quinine HCL [54]. In some cases the micelle mobility may vary somewhat depending on the nature of the incorporated compound. Therefore it may be possible in certain instances that the mobility of the solute-micelle complex may not be exactly the same as the mobility of the marker-micelle complex. In the α-cyclodextrin systems, estimation of μ_c at high α-cyclodextrin concentrations (from Fig. 6)

Table 4
Values of the electrophoretic mobility of the solute–ligand complex estimated graphically using Eqs. 12–14[a]

Compound	μ_c (cm^2/kV min)		
	Double reciprocal method and y-reciprocal method	x-Reciprocal method	Experimental values[b]
SDS system			
p-Nitrophenol			
not adjusted[c]	−27.9 ± 2.7	−33.2 ± 3.1	−22.8 ± 0.3
adjusted[d]	−36.0 ± 13	−36.0 ± 20	−24.9 ± 0.3
p-Nitroaniline			
not adjusted	−27.9 ± 0.4	−27.8 ± 0.3	−22.8 ± 0.3
adjusted	−30.6 ± 1.3	−29.4 ± 2.3	−21.4 ± 0.3
Hydroquinone			
not adjusted	−18.2 ± 0.8	−18.0 ± 0.3	−22.8 ± 0.3
adjusted	−22.4 ± 2.2	−22.3 ± 0.7	−23.6 ± 0.3
Naphthalene			
not adjusted	−24.4 ± 1.4	−22.9 ± 1.5	−22.8 ± 0.3
adjusted	−21.9 ± 0.8	−23.0 ± 4.1	−21.4 ± 0.3
α-Cyclodextrin system			
p-Nitrophenol	−5.8 ± 0.1	−5.8 ± 0.2	−6.4[e]
m-Nitrophenol	−6.2 ± 0.2	−6.4 ± 0.6	−9.2[e]

[a] See Experimental section for further details.
[b] Experimental values were estimated with quinine-HCl as the micelle marker.
[c] Data was not adjusted for viscosity changes prior to analysis.
[d] Data was adjusted using a neutral marker. See Table 2 for more details.
[e] Estimated from Fig. 6 as the mobility at the highest α-cyclodextrin concentration.

Table 5
Binding constants and enantioselectivities for 2-phenoxypropionic acid and 2-(4-chlorophenoxy)propionic acid to hydroxypropyl-β-cyclodextrin in 50 m*M* phosphate, pH 7.0 buffer at 23°C

Plotting method	K(1)[a]	K(2)[b]	α[c]	$\mu_c(1)$[d]	$\mu_c(2)$[d]
2-Phenoxypropionic acid					
Mobility ratio[e]	–	–	–	–[e]	–[e]
Double reciprocal	31.2 ± 2.5	27.8 ± 3.1	1.12	−4.6 ± 0.3	−4.4 ± 0.4
y-reciprocal	31.2 ± 2.5	27.8 ± 3.1	1.12	−4.6 ± 0.3	−4.4 ± 0.4
x-reciprocal	31.7 ± 2.8	26.8 ± 3.4	1.18	−4.7 ± 0.4	−4.1 ± 0.5
2-(4-Chlorophenoxy)propionic acid					
Mobility ratio[f]	153 ± 15	133 ± 10	1.15	−5.9[f]	−5.9[f]
Double reciprocal	113 ± 14	111 ± 15	1.02	−5.0 ± 0.4	−5.4 ± 0.4
y-Reciprocal	113 ± 14	111 ± 15	1.02	−5.0 ± 0.4	−5.4 ± 0.4
x-Reciprocal	101 ± 12	100 ± 16	1.01	−4.5 ± 0.6	−4.9 ± 0.9

[a] Binding constant (M^{-1}) for the first eluting enantiomer.
[b] Binding constant (M^{-1}) for the second eluting enantiomer.
[c] Enantioselectivities (α) were calculated as: $\alpha = K_1/K_2$.
[d] Electrophoretic mobilities of the enantiomer:cyclodextrin complex in (cm^2/kV min) for the 1st and 2nd eluting enantiomers.
[e] This method was not used because the complex mobilities cannot be estimated from Fig. 11.
[f] The complex mobilities were taken as the measured mobility at the highest hydroxypropyl-β-cyclodextrin concentration (see Fig. 11). This value is used as an estimate only.

resulted in gross overestimation of μ_c for both *m*-nitrophenol and *p*-nitrophenol (see Table 4).

Table 5 lists the binding constants and enantioselectivities calculated using Eq. 1 and Eqs. 12–14 for chiral compounds to hydroxypropyl-β-cyclodextrin. The enantioselectivities (α) were calculated from the binding constants: $\alpha = K_1/K_2$. The mobility ratio method was used for binding constant estimation for 2-(4-chlorophenoxy)propionic acid using the mobility measured at the highest cyclodextrin concentration as μ_c. The values given with this method are quite different than those given by the other methods which do not require the knowledge of μ_c. Clearly the enantioselectivity and binding constants are grossly overestimated by the mobility ratio method when μ_c cannot be accurately measured. Suitable markers are not available for cyclodextrin systems and saturating conditions often cannot be reached at concentrations lower than the solubility. For a given compound, the μ_c values calculated from the various plotting methods (Eqs. 12–14) are all in agreement within experimental error. In addition, μ_c values for each enantiomer of an enantiomeric pair are the same within experimental error. While it has been reported that enantioresolution may partially result from different μ_c values for each enantiomer–cyclodextrin complex [69], it does not appear to be the case for these compounds.

These linear methods (Eqs. 12–14) are advantageous for systems where the mobility of the solute–ligand complex is not known and cannot be adequately measured with a marker or at saturating concentrations of ligand. While Eqs. 12–14 are mathematically identical to Eq. 1, they are not necessarily experimentally equivalent. Eq. 1 can be used only when μ_c can be measured experimentally. However, suitable markers are currently available only for micellar systems [40,41,54]. For the systems, such as cyclodextrins, proteins, antibiotics, etc., μ_c usually cannot be accurately measured. In these cases, Eq. 1 cannot be used. Instead, either non-linear curve fitting [42–44] or the linear curve fitting data analysis methods outlined above can be employed.

These methods are simple, linear graphical methods for the estimation of binding constants from data collected using capillary electrophoresis. A unifying approach is presented for all

the relationships used to calculate binding constants in affinity electrophoresis, CE micellar electrokinetic separations, as well as other systems. These methods can be used for the estimation of apparent binding constants for any type of ligand, including but not limited to micelles, chiral selectors (cyclodextrins, crown ethers, antibiotics, etc.) and proteins. They produce reasonable results with acceptable error limits. Historically, linear methods have been preferred for the analysis of other types of experimental data. The high efficiency and ease of capillary electrophoresis combined with these plotting methods makes the estimation of binding constants using capillary electrophoresis a simple, straight forward process.

4. Conclusion

The complete family of general linear plotting methods are presented for the determination of binding constants using capillary electrophoresis. The close relationship between these expressions and the advantages and disadvantages of these approaches are discussed. For optimum results, the plotting methods should be used in conjunction with weighted least squares linear regression. It should be emphasized that these plotting methods for CE are analogous to those which have been used for a variety of analytical techniques, including spectroscopy, calorimetry and chromatography. They belong to a large family of equations that have been used to quantitate equilibrium processes for many years. The equations are obtained by comparison of the fundamental electrophoretic relationship between solute mobility and ligand concentration to the general form of the binding isotherm. The value of the electrophoretic mobility of the complex formed by the solute and ligand is not required in order to use these methods.

Acknowledgements

Support of this work by the Department of Energy, Office of Basic Sciences (grant DE FG02 88ER13819) is gratefully acknowledged. Thanks are extended to Dr. Nuran Ercal for the use of the P/ACE 2100 capillary electrophoresis unit and to Dr. Gary L. Bertrand for helpful discussions and for providing the Least Squares software.

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