Capillary Electrophoresis as a Method for Determining Binding Constants: Application to the Binding of Cyclodextrins and Nitrophenolates†

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For analytes involved in dynamic equilibrium processes, capillary electrophoresis is a powerful method of determining binding constants. Equilibrium constants from capillary electrophoresis for the binding of nitrophenolates to α-cyclodextrin show good agreement with literature values obtained using calorimetric and spectroscopic methods, confirming capillary electrophoresis as a viable method. We show that it is imperative to make viscosity corrections, to study the full binding range, and to use an algorithm which calculates the concentration of free cyclodextrin rather than the total cyclodextrin concentration. Binding constants for analytes in a complex mixture can be determined simultaneously. The methods have been applied to 2-, 3-, and 4-nitrophenolates with native and derivatized α- and β-cyclodextrins, and results provide insight into the binding process. Data analysis methods for capillary electrophoresis are also successfully applied to liquid chromatography with the use of the same selector as mobile phase additive.

Introduction

Much work has been carried out into the study of cyclodextrin (CD) inclusion complexes. CDs are toroidally-shaped polysaccharides formed from D-glucose monomers. The α-, β-, and γ-CDs consist of 6, 7, and 8 glucose units, respectively. They have a hydrophobic cavity and a hydrophilic exterior which gives rise to their ability to form complexes with a wide range of small molecules. In aqueous solution, CDs form inclusion complexes into their cavity through hydrogen-bonding, van der Waals forces, and hydrophobic interactions.[1] The present investigation is concerned with the reaction between CDs and nitrophenolates (NP^-) for which structural evidence for inclusion complex formation is available from X-ray crystallography.[2,3]

The first systematic study of complexation equilibria between nitrophenols (NP), nitrophenolates and cyclodextrins was by Cramer *et al.*,[4] using UV spectrophotometry to monitor spectral shifts on complexation and Hildebrand Benesi plots to analyse the data to obtain equilibrium constants. Inoue *et al.*[5] used nuclear magnetic resonance (NMR) and monitored the ^1H chemical shift of the NP^- as a function of [CD]. They also used a least-squares fitting of a modified Hildebrand Benesi plot. Bertrand *et al.*[6] measured the equilibrium constants and enthalpy changes by "high dilution" reaction calorimetry, for the formation of complexes of α- and β-CD with substituted phenols and nitrophenolate ions. This very thorough study included variation of the size and shape of the guest molecule, the degree of methylation of the CDs, and the effects of pH and ionic strength.

For an analyte involved in a dynamic guest:host equilibrium process, capillary electrophoresis (CE)[7-9] can provide an alternative method for determining binding constants through determination of the electrophoretic mobility as a function of the concentration of the host molecule.[10,11] The aims of the present work are to validate the CE method using a NP^-:CD complex where the equilibrium constant has been accurately determined in previous studies and to use the unique features of CE to simultaneously determine binding constants for a set of analytes in a complex mixture. Analogous work has been reported comparing binding of analytes to micelles by CE and other methods.[12-14]

Theory

CE is an analytical technique for the separation of water-soluble analytes using the principle of the electrically driven flow of ions in solution, under a high-voltage gradient.[7] The most common mode of performing CE is free solution CE. In this technique the analytes, which are dissolved in an aqueous buffer, are separated according to their electrophoretic mobility, μ, which in turn depends upon charge-to-size ratio

$$\mu = q/6\pi\eta r \qquad (1)$$

where q is the charge, r the radius, and η the viscosity.[15] Electroosmotic flow (EOF), which arises due to the negatively-charged walls of the fused silica CE capillary, carries the bulk of the liquid along the capillary. This normally causes all species to have a net migration toward the cathode and past the detector regardless of the sign of μ.

In the case of the CD:NP^- complexes studied, the CDs are uncharged and thus migrate under the influence of the EOF only. The NP^- ions all have a charge of -1 under the conditions of these experiments (pH 11.1) and have an electrophoretic mobility, μ_0, of negative sign since pK_a values are 7.2, 8.4, and 7.2 for 2-, 3-, and 4−NP, respectively.[16] A fully bound CD:NP^- complex also carries a charge of -1 but is considerably bulkier, causing it to have an electrophoretic mobility, μ_∞, of a lower magnitude than μ_0. When NP^- is injected as analyte with CD as the selector present at constant concentration in the run buffer, the observed electrophoretic mobility, μ, lies between these two extremes and indicates the fraction of time the NP^- ion spends in the uncomplexed form and the fraction of time it spends bound to the CD. Because these systems are in dynamic equilibrium, analysis of mobility allows direct measurement of binding constants.

$$\frac{\mu_0 - \mu}{\mu - \mu_\infty} = \frac{[\text{CD:NP}^-]}{[\text{NP}^-]} \qquad (2)$$

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Since the binding constant K is given by

$$\frac{[CD:NP^-]}{[CD][NP^-]} = K \quad (3)$$

$$\frac{\mu_0 - \mu}{\mu - \mu_\infty} = KC \quad (4)$$

where C is the concentration of free cyclodextrin.

Electrophoretic mobility is calculated using eq 5,

$$\mu = \frac{Ll}{V}\left(\frac{1}{t} - \frac{1}{t_0}\right) \quad (5)$$

where L is the length of the capillary, l the length of the capillary to the detector, V the voltage, t the migration time of the analyte, and t_0 the migration time of the electroosmotic flow.[15] Mobilities from eq 5 are normally corrected for viscosity variation prior to data fitting, as discussed in subsequent sections. By determining μ_0 and the mobility of NP^- at a number of different CD concentrations, a series of data points can be plotted.[17] A non-linear least-squares fit is used to fit the data, with K and μ_∞ as variables. The optimum value of K is found by minimizing χ^2, the measure of the goodness of fit.[18] A Microsoft Windows based PC program, CEFit, has been written in house to calculate binding constants. The CEFit program will fit data for systems in equilibria involving a single analyte species or a pair of enantiomers, with and without a competitor present. Equation parameter values are fixed or fitted, where appropriate, by means of a grid search. Data is input from a spreadsheet (ASEASYAS). The results are displayed in graphical and tabular form, and can be output back to the spreadsheet for further manipulation.

This method of determining binding constants by CE can be applied to any system as long as the following criteria are met.[19]

(i) The time scale of equilibration is faster than the time scale of the separation being performed.

(ii) There are significant proportions of both selector (in our case unbound CD) and selector:analyte complex in the capillary.

(iii) The mobility of the analyte and complex are different. In CE this involves the selector or analyte being charged.

In free solution CE complexes between neutral species cannot be investigated. In the present work this limits us to a study of the $CD:NP^-$ complex rather than both CD:NP and $CD:NP^-$ complexes. The equations described here are appropriate for equilibria involving specific binding. Similar criteria hold for partition equilibria, for example binding into micelles, and data treatment methods have been discussed elsewhere for both neutral[12,13] and ionic species.[20]

Experimental Section

CE experiments were carried out on an automated system (Beckman, P/ACE 2100), thermostatted at 25.0 °C. Each experiment was run in triplicate, with mesityl oxide as a neutral marker. Relative viscosity was determined by taking the ratio of the currents I_0 at [CD] = 0, and I at [CD] = C ($I_0/I = \eta/\eta_0$). Hydroxypropyl-β-CD and methyl-β-CD were gifts from Wacker Chemicals (Halifax, UK). All other materials were from Aldrich (Gillingham, UK). The fused-silica separation capillary had an internal diameter of 50 μm, a total length of 57 cm and a length of 50 cm from inlet to detector. A voltage of 15 kV was used for the separation, and detection was at 230 nm. The pH 11.1 run buffer was prepared by titrating 100 mM phosphoric acid with 5 M sodium hydroxide, and diluting 2-fold, and adding a specified concentration of cyclodextrin. The samples were loaded by a 1 s pressure injection (corresponding to an injection volume of 1 nL) dissolved in run buffer at various concentrations as stated.

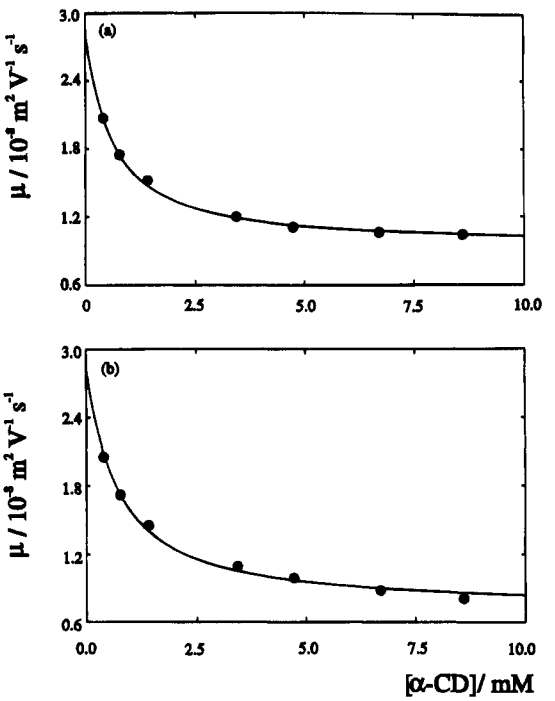

Figure 1. Electrophoretic mobility of 3-nitrophenol as a function of [α-CD]. (a) Data corrected for viscosity variation as discussed in Experimental Section and fitted to binding equilibrium curve, giving $K = 199 \pm 5\ M^{-1}$, $\mu_\infty = (0.93 \pm 0.01) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$. (b) Data uncorrected for viscosity variation, and data fitted to binding equilibrium curve, giving $K = 165 \pm 5\ M^{-1}$, $\mu_\infty = (0.71 \pm 0.01) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$. Buffer, aqueous 50 mM phosphate; pH 11.1; temperature 25 °C; voltage 15 kV; capillary, 50 μm internal diameter, 57 cm length; detection, 230 nm; injection, 1 nL; [3-NP^-], 1.4 mM.

The CE buffer conditions were chosen to be identical to those used by Bertrand *et al.*,[6] to enable comparisons between techniques to be made without corrections for differences in pH and ionic strength. In the case of the conditions used by Cramer *et al.*,[4] results were interpolated to 25 °C by Bertrand *et al.*[6]

The LC system consisted of a ternary gradient pump (ACS, Model 352), an injection valve (Rheodyne 7152) with a 20 μL loop, and a variable wavelength UV detector (ACS, 750/12) operating at 254 nm. The UV data were collected and analysed on an integrator (Trivector Trio). A C_{18} column (5 μm particle size, ODS2, 250 × 4.6 mm i.d.; HPLC technology, Macclesfield, UK) was maintained at 25 °C using a thermostatted water jacket. The mobile phase was pH 8.5, 200 mM phosphate buffer, containing a specified concentration of cyclodextrin, at a flow rate of 0.7 mL min^{-1}.

Results and Discussion

(A) Data Handling. The mobility of $3\text{-}NP^-$ was measured to be $\mu_0 = 2.82 \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$, and mobilities as a function of α-CD concentration are given in Figure 1a. The binding curve given in Figure 1a is fitted with K and μ_∞ as variables according to the method described in the theory section, giving $K = 199 \pm 5\ M^{-1}$, and $\mu_\infty = (0.93 \pm 0.01) \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$. When processing data obtained from CE for the determination of binding constants there are three important issues that need to be considered.

(i) Variation of Viscosity. Solutions containing high concentrations of cyclodextrins become very viscous, and since μ

TABLE 1: Variation of K and μ_∞ for the α-CD:3-NP⁻ Complex with Assumed Values of NP⁻ Concentration

[3-NP⁻]/M	K/M^{-1}	μ_∞/10^{-8} m^2 V^{-1} s^{-1}
0	173 ± 3	0.91 ± 0.01
0.0014[a]	199 ± 5	0.93 ± 0.01
0.003	242 ± 10	0.96 ± 0.01

[a] Refers to correct value of [NP⁻].

$\propto 1/\eta$ (eq 1) failure to recognize this can result in the misinterpretation of data, as has been previously pointed out.[11,17,21] η/η_0 is a power series in concentration,[22] and therefore it is especially important to make viscosity corrections when determining K for weakly-bound species, which require high [CD] at half maximum binding. Buffer viscosity changes affect the mobility of all the ionic species. Since the current in the CE capillary is directly proportional to mobilities, a correction for viscosity variation can be made by measuring the current during CE experiments as discussed in the Experimental Section. This eliminates the need to independently measure the viscosity. Figure 1b shows values obtained for mobilities without correction for viscosity variation and the fitted binding curve, which gives values of $K = 165 \pm 5$ M^{-1}, and $\mu_\infty = (0.71 \pm 0.01) \times 10^{-8}$ m^2 V^{-1} s^{-1}. It should be noted how K and μ_∞ are underestimated without correction. When $K \leq 100$ M^{-1} the mobility change with [CD] at high CD concentration due to viscosity variation will typically be greater than that due to binding.

(ii) Cyclodextrin Concentration. A second factor to consider is the need to calculate K using the *free* CD concentration and not just the *total* concentration, especially when the binding constant is high. The algorithm we employ calculates this as each point.[23] In the example discussed here, the concentration of 3-NP⁻ injected onto the capillary is 1.4 mM, and this is taken as the value throughout the experiment since in CE there is effectively no dilution on the capillary at the peak maximum. It is clearly important to specify the value of analyte concentration where possible. When an incorrect value of [NP⁻] is used, this leads to incorrect calculation of the *free* CD concentration, in turn leading to systematic error in determination of K and μ_∞. Table 1 shows typical errors that can be observed through the use of the incorrect value of [NP⁻]. Because of the need to use the *free* CD concentration it is also important to make sure that [CD] ≫ [NP⁻]. This is particularly crucial when K is large, and the half maximum binding cyclodextrin concentration is small, where large injection concentrations will lead to erroneous results.

When measuring binding constants it is imperative to work in the correct [CD] range around $C = K^{-1}$ in order to fully describe the binding curve. For example, for 3-NP⁻ with α-CD, $K = 199$ M^{-1}, therefore [CD] at half-maximum binding is 5 mM. An ideal range has concentration changing by at least one decade. With very high binding constants, this can cause problems as discussed previously. With very low binding constants limits of solubility of the cyclodextrin may limit the working range possible.

(iii) Temperature Increase. The third important factor to be aware of when using CE to derive equilibrium data is the temperature increase, ΔT, in the capillary from Joule heating. Capillaries must be thermostated by forced air cooling or liquid cooling to avoid high internal temperatures. ΔT is dependent on the power dissipation in unit length, $P = IV/L$, typically 0.1−5 W m^{-1} in CE experiments. The relationship between power dissipation and solution temperature has been discussed by several groups.[24,25] Either mobility data and equilibrium constants should be determined at a series of powers and extrapolated to zero[26] or the dependence of ΔT and P measured for the particular CE instrument/capillary combination using a well characterised calibration solution.[27] For the liquid cooled CE capillary in this study with $I = 60 \pm 3$ μA, $P = 1.58 \pm 0.08$ W m^{-1}, calibration using the mobility of benzoate ion[27] gave $T = 27.8 \pm 0.5$ °C for a set temperature of 25.0 °C, an average solution temperature increase of 2.8 °C relative to the external surface of the capillary.

It should be noted that systematic errors in the neglect of viscosity and temperature corrections combine to underestimate K by 26%. These data handling issues are of wide applicability in using CE to study a variety of host:guest and selector:selectand equilibria, for example in analyzing data for enantiomeric separations,[23] and for the emerging technique of affinity CE.[28]

(B) Applicability of the CE Method. Values for K, μ_o, and μ_∞ for 3- and 4-NP⁻ binding to α- and β-CD obtained from CE before temperature correction are presented in Table 2. The table also shows the binding constants corrected to 298 K, along with results obtained using other techniques.[4−6] The key point to note from Table 2 is that the values for 3-NP⁻ binding to α-CD and β-CD, and for 4-NP⁻ binding to α-CD are in good agreement with those determined by calorimetric and spectroscopic methods. This confirms the applicability of CE as a method for determining binding constants.

For both α-CD and β-CD, equilibrium constants are an order of magnitude greater with 4-NP⁻ than with 3-NP⁻. For α-CD, X-ray studies of the neutral 4-NP⁻:CD complex show the NO_2 group situated on the primary O(6) side and hydrogen bonded to water, while the OH group protrudes from the secondary hydroxyl side. It can be assumed that with 4-NP⁻ there is a similar good fit, whereas 3-NP⁻ gives a much worse fit into the cavity in terms of meeting both steric and polarity requirements. Table 2 suggests some controversy in the literature as regards the value of the equilibrium constant for the binding of 4-NP⁻ to α-CD, which the present study helps to resolve. The figure of 769 M^{-1} obtained using NMR[5] is seen to be very low. We believe this is a consequence of the use of inappropriate concentrations of 4-NP⁻ and α-CD. Due to the relatively large concentration of 4-NP⁻ needed to obtain an NMR signal, [NP⁻] was kept constant at 10 mM, while [α-CD] was varied from 0.25 to 50 mM. Following considerations in the previous section the binding curve cannot have been correctly established since the analyte concentration is in excess of the selector concentration over much of the range and also higher than the concentration of selector for half-maximum binding.

(C) Simultaneous Binding Constant Determination. Due to the ease of automation and nature of the separations in CE, binding constants for several analytes can be determined simultaneously. Figure 2 shows the electropherograms of the 2-, 3-, and 4-NP⁻ at various concentrations of α-CD, with Figure 3 showing the binding curves obtained from these data. In Figure 2 the effect of viscosity variation is evident in the electropherogram where [α-CD] = 20 mM, with the mesityl oxide peak (marker for the electroosmotic flow) shifting by almost a minute. Note that all the NP⁻ species migrate *after* the neutral marker, because they are negatively charged at the pH of the experiment but are still migrating past the detector due to the EOF.

From Figures 2 and 3, it can be seen that the binding constant of the α-CD:2-NP⁻ complex is very low, and the fitted value is $K = 11 \pm 0.2$ M^{-1}. Since the CD concentration at half-maximum binding is calculated to be 83 mM, this highlights the problem identified in the section on data fitting. It is impractical to work at significantly higher concentrations to fully

TABLE 2: Values of Electrophoretic Mobilities and Binding Constants for Binding of 3- and 4-NP⁻ to a Series of Native and Derivatized α- and β-CDs

NP⁻	CD	K/M^{-1}	$\mu_0/10^{-8}$ m² V⁻¹ s⁻¹	$\mu_\infty/10^{-8}$ m² V⁻¹ s⁻¹	μ_∞/μ_0	K_{298}/M^{-1} CE	K_{298}/M^{-1} lit.
3−	α	199 ± 5	2.82	0.93 ± 0.01	0.33	224 ± 11	202 ± 3[a]
4−	α	1570 ± 20	2.94	0.93 ± 0.01	0.32	1830 ± 63	1800 ± 300[a] 2290[b] 769[c]
4−	HP-α	1862 ± 91					
3−	β	109 ± 3	2.84	1.01 ± 0.02	0.35	112 ± 4	117 ± 19[a]
4−	β	1503 ± 165	2.94	0.95 ± 0.01	0.32	1593 ± 192	570 ± 25[a]
4−	HP-β	654 ± 99					
4−	Me-β	677 ± 32					

[a] Reference 6. [b] Reference 4. [c] Reference 5.

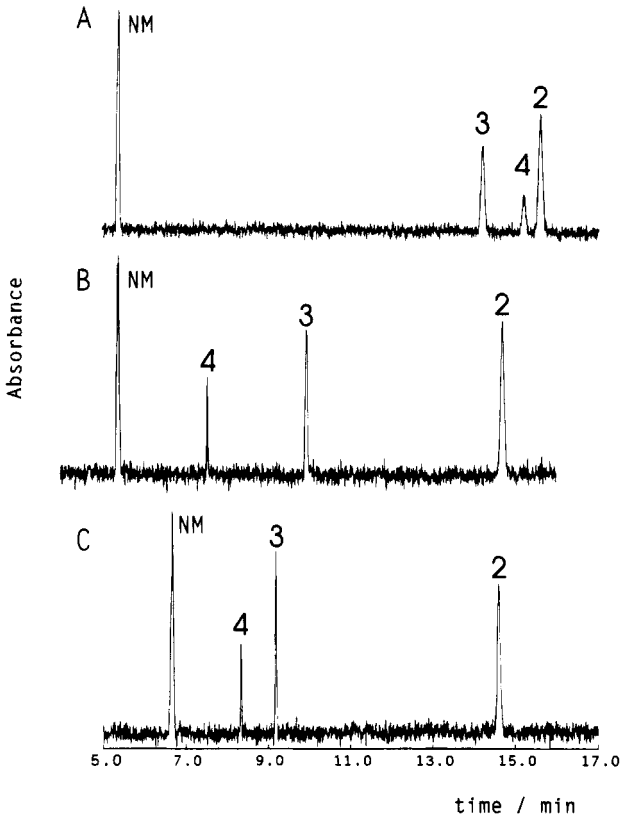

Figure 2. Electropherograms of a mixture of 2-, 3-, and 4-nitrophenolate as a function of α-CD concentration. (A) no cyclodextrin, (B) 4 mM α-CD, (C) 20 mM α-CD. NM = mesityl oxide as neutral marker. Separation and buffer conditions as Figure 1.

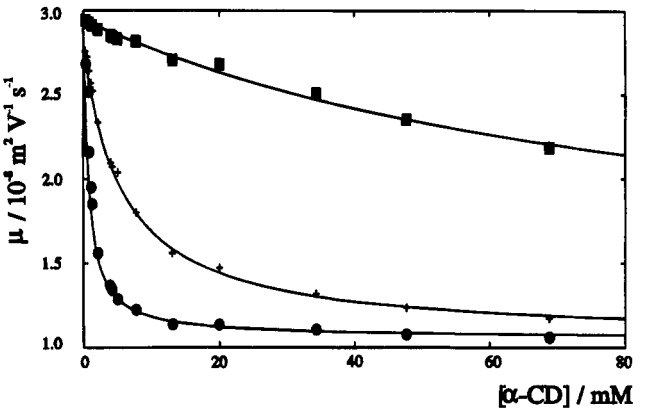

Figure 3. Electrophoretic mobilities of 2-, 3-, and 4-nitrophenolates as a function of [α-CD], simultaneously measured. Data fitted to equilibrium curves giving: 2-NP⁻, (filled squares), $K = 11 \pm 0.2$ M⁻¹, $\mu_\infty = (0.99 \pm 0.03) \times 10^{-8}$ m² V⁻¹ s⁻¹: 3-NP⁻ (crosses), $K = 180 \pm 2$ M⁻¹, $\mu_\infty = (0.92 \pm 0.01) \times 10^{-8}$ m² V⁻¹ s⁻¹: 4-NP⁻ (filled circles), $K = 1500 \pm 18$ M⁻¹, $\mu_\infty = (0.95 \pm 0.01) \times 10^{-8}$ m² V⁻¹ s⁻¹. Analyte concentration: [2-NP⁻] = 0.14 mM; [3-NP⁻] = 0.095 mM; [4-NP⁻] = 0.086 mM. Separation and buffer conditions as Figure 1. Data uncorrected for temperature effects.

describe the curve, due to the large viscosity variations and maximum solubility of α-CD (150 mM). However, we have confidence in the quality of the value of K since the other variable $\mu_\infty = (0.99 \pm 0.01) \times 10^{-8}$ m² V⁻¹ s⁻¹ for α-CD: 2-NP⁻ complex is comparable to that for the other complexes, and the mobility ratio μ_∞/μ_0 of 0.33 is identical to values reported in Table 2 for the the binding of 3- and 4-NP⁻ with α-CD. μ_∞/μ_0 has previously been shown to be the appropriate parameter characterizing size change on going from the guest to the host:guest inclusion complex.[29]

While the mixture separated in Figure 2 was prepared from standards, CE does allow the measurement of binding constants for species in a mixture where any excipients or contaminants that are UV absorbing would make spectrophotometric measurements impossible. It is also possible that impure mixtures of pharmaceutical compounds could be screened for potential pharmacological activity, without the need for development of expensive purification and scale-up methods.

(D) A Study of 4-Nitrophenolate Binding to β-Cyclodextrin. In Table 2 it can be seen that the value of K from CE for 4-NP⁻ binding to β-CD is not consistent with the value given in reference.[6] Data points were found to deviate from the 1:1 binding curve, and we postulate in this experiment that two CDs are complexing a single 4-NP⁻, hence leading to a bad fit of the data. Armstrong *et al.*[30] show evidence for a single 4-nitroaniline complexing two β-CDs, and they suggest that the situation of one substrate binding two CDs is possibly more widespread than the literature indicates and that great care must be taken when interpreting data. They also note that it is possible that one CD is forming the inclusion complex, while the other CD is hydrogen bonding to the substrate near the cavity rim. This would affect the CE mobility of the complex, and thus the binding of the second CD must be very weak, otherwise we would expect to observe a much smaller value of μ_∞. The limit of solubility for β-CD, 16 mM, meant that as discussed in section A(ii), we were unable, using a modification of the CEFit program to fit both 1:1 and 2:1 complexes concurrently, to extract a value of the binding constant for the second CD.

The effect of derivatization of cyclodextrins on the binding constant with 4-NP⁻ is documented in Table 2. Hydroxypropyl-α-CD (HP-α-CD) has a higher value of K for binding to 4-NP⁻ than the native α-CD. Hydroxypropylation introduces a bulky group with a hydrophilic center, which we postulate interacts

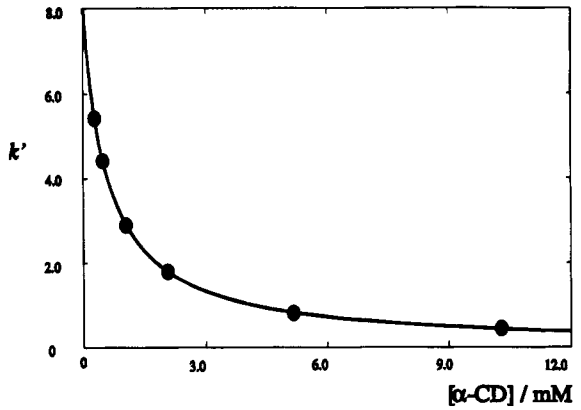

Figure 4. Retention factor of 4-nitrophenolate as a function of [α-CD], and data fitted to binding equilibrium curve, giving $K = 1720 \pm 40$ M^{-1} and $k'_\infty = 0.01 \pm 0.03$. Buffer 200 mM phosphate, pH 8.5, [4-NP^-] 5 mM; other conditions as in Experimental Section.

with the nitrophenolate, and aids binding. This phenomenon has been observed when studying other compounds.[23] HP-β-CD and methylated β-CD (Me-β-CD) give values of K of the same order of magnitude as reported for the native β-CD in reference 6, and the binding curves show no evidence of poor fitting. Therefore, it is possible that derivatization around the cavity rim in the HP-β-CD and Me-β-CD poses a steric hindrance to the second CD binding by 4-NP^-.

(E) Applicability to Liquid Chromatography. Studies in our laboratory have also been concerned with the applicability of data analysis for CE to data analysis for high performance liquid chromatography (HPLC) with mobile phase additives.[31] Equation 4 can be compared to a similar equation in HPLC, where retention factors (k')[32] are substituted for mobility values.[31]

$$KC = \frac{k'_0 - k'}{k' - k'_\infty} \qquad (6)$$

A binding curve analogous to that calculated with mobility data can therefore be constructed, where k'_0 is the retention factor of the free analyte, k'_∞ the retention factor of the selector:analyte complex, and k' the retention factor at a particular concentration of selector. Figure 4 shows the fitted binding curve to the retention factor data for 4-NP^- binding to α-CD. This uses the computer program as for CE, with substitution of k' values for mobilities giving $K = 1720 \pm 40$ M^{-1}, with $k'_0 = 0.01 \pm 0.03$. This is in satisfactory agreement with the data at 298 K (Table 2) from CE (1830 ± 63 M^{-1}) and calorimetry[6] (1800 ± 300 M^{-1}). The HPLC experiments were carried out at pH 8.5, as the pH of the CE experiments (pH 11.1) is outside the recommended range of the C_{18} column. However, pH is not expected to affect K over this range because the analyte remains negatively charged (pK_a for 4-NP = 7.2). The lower value of K from HPLC than from CE can be attributed to differences in ionic strength. As solutions used in the calorimetry and HPLC studies (ionic strength 0.9 and 0.6 mol kg^{-1}, respectively) gave unacceptably high currents in CE, the ionic strength was set at 0.15 mol kg^{-1} for the CE experiments. Correction for nonideality using Debye–Hückel theory[23,33] gives the thermodynamic equilibrium constant, K°, 2049 ± 48 (HPLC), 2018 ± 70 (CE), and 2196 ± 366 (calorimetry), which are now in excellent agreement.

Use of the treatment of equation 6 is appropriate when there is no binding of the cyclodextrin to the stationary phase and $k'_\infty = 0$.[34] This is shown to be valid since the value of k'_∞ estimated by the fitting program equals zero within experimental error. Due to the very high on-column dilution obtained in HPLC and partition of the analyte between mobile and stationary phase, the analyte concentration in the mobile phase as low selector concentration is 2 orders of magnitude lower than that injected. Corrections in the Data Handling, subsection (ii), are thus expected to be less important in HPLC experiments using mobile phase additives than in CE.

In contrast to CE, HPLC is able to measure binding constants where both analyte and selector are neutral. While the measurement of binding constants by HPLC has previously been reported,[34] our approach of using a unified data analysis scheme for CE and HPLC is novel.[31]

Conclusions

CE is found to be an excellent method of determining binding constants, and consistency with values from spectrophotometry and calorimetry confirms the applicability of the technique. We highlight the need to apply corrections for viscosity change, to calculate the free cyclodextrin concentration, to use the appropriate cyclodextrin concentration range, and to be aware of temperature change in the capillary from Joule heating. The methods described here can be applied to systems other then host:guest complexes of cyclodextrins and charged analytes, as long as the three criteria mentioned in the Introduction are adhered to. In summary, (i) the time scale of equilibration must be faster than the time scale of the separation being performed, (ii) there are significant proportions of both selector (in our case unbound CD) and selector:analyte complex in the capillary and, (iii) the mobility of the analyte and complex are different; in CE this involves the selector or analyte being charged. The use of CE to determine binding constants simultaneously for a mixture of analytes offers an advantage over other techniques such as calorimetry and spectrophotometry. These methods are expected to be widely applicable, for example in studying enzyme:substrate, protein:drug, and metal:ligand binding.

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