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Separation of positional and structural isomers by cyclodextrin-mediated capillary zone electrophoresis

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Abstract

Previously derived models for optimization of cyclodextrin (CD)-mediated capillary zone electrophoresis (CZE) referred only to the separations of enantiomers. These models assume that the mobility of the inclusion complexes of the two solutes are equal (i.e., $\mu_{ACD}=\mu_{BCD}$). With other types of solutes, such as positional and structural isomers, this assumption is not valid (i.e., $\mu_{ACD}\neq\mu_{BCD}$). In this work, the effectiveness of the model of Wren and Rowe, which was developed for enantiometric separations, is evaluated for cyclodextrin-mediated CZE of other types of solutes. Experimental data is obtained for the α-cyclodextrin-mediated separation of positional and structural isomers, modelled by nitrophenols and phenylbutyric acids, respectively. It was found that the mobilities of the inclusion complexes of the isomers differed from one another ($\mu_{ACD}\neq\mu_{BCD}$) and that the complex mobility did not correlate with the solute mobility, the formation constant or the "quality of fit". Despite the complex mobilities for the positional and structural isomers not being equal, the Wren and Rowe model is nonetheless effective for predicting the optimum α-cyclodextrin concentration. Only when the formation constants for two isomers are approximately equal ($K_{ACD}\approx K_{BCD}$) does the optimum α-cyclodextrin concentration differ from that predicted.

Keywords: Positional isomers; Structural isomers; Buffer composition; Cyclodextrins; Nitrophenols; Phenyl butyrates

1. Introduction

Cyclodextrins are widely used in the separation of enantiomers by capillary zone electrophoresis (CZE) [1–6]. While not as common, cyclodextrins are also used to optimize the separations of peptides [7], nucleosides and nucleotides [8], and positional isomers [9,10]. With such solutes, secondary equilibria such as acid–base dissociation [11] and micellar partitioning [12] can also be used to optimize separations. However, such approaches are not always successful. It is therefore desirable to have a fundamental understanding of the factors that govern the separation of achiral solutes in cyclodextrin-mediated CZE. Unfortunately, models of the effect of cyclodextrin complexation were derived to describe enantiomeric separations [13–18]. Thus, their applicability to the cyclodextrin-mediated CZE separation of other classes of solutes is not clear. In this work, we investigate the factors that affect the optimization of separations of positional and structural isomers, as model achiral solutes.

2. Experimental

2.1. Apparatus

All experiments were performed on a Beckman P/ACE 2100 CE instrument (Fullerton, CA, USA).

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The capillary was 47 cm long fused-silica (Polymicro, Phoenix, AZ, USA) with an internal diameter of 75 μm. The UV absorbance detection was on-capillary, 7 cm from the cathode end. Direct detection at 254 nm was used for the nitrophenolates and 214 nm was used for the phenyl butyrates. All capillaries were pretreated with 0.1 *M* sodium hydroxide for 10 min, water for 5 min and with buffer solution (Tris or phosphate), prior to use. Data acquisition and instrument control were performed using System Gold software (Beckman) operating on a 386-based microcomputer.

2.2. Reagents

Ultra-pure Tris (Schwarz/Mann), assurance-grade phosphate (BDH) and distilled deionized water (Barnstead Type D4700 NANOpure Deionization System) were used in the preparation of buffer and sample solutions. All buffer solutions prepared contained either 20 m*M* Tris (pH 7.10) or 10 m*M* sodium phosphate (pH 11.1). Solute molecules were obtained as solids from Aldrich and were prepared at concentrations of $5 \cdot 10^{-5}$ *M* in the buffer. Cyclodextrins were obtained from Amaizo (American Maize-Products Company, Hammond, IN, USA). Mesityl oxide ($5 \cdot 10^{-5}$ *M*; Aldrich) was added to sample solutions as an electroosmotic flow marker. Cyclodextrin was added to buffer solutions, but not to sample solutions. Hydrochloric acid and sodium hydroxide were used to adjust all solutions to the desired pH ranges. All solutions were filtered through 0.45 μm filters prior to use and stored in nalgene bottles. Solutions containing cyclodextrin were used within two days of preparation.

2.3. Procedures

All separations were performed at 15 kV. No significant Joule heating was observed under these conditions, as confirmed by Ohm's law plots for each buffer. Sample solutions were injected using pressure injection for 1 s for each trial. After each run, the capillary was rinsed first with 0.1 *M* sodium hydroxide for 1 min, next with water for 1 min and finally with buffer solution for 2 min.

The viscosity of the electrophoretic buffer was measured by monitoring the time required for an injection of mesityl oxide to reach the detector window when low pressure (5 p.s.i.; 1 p.s.i.= 6894.76 Pa) was applied to the inlet of the capillary [8].

2.4. Calculations

The electrophoretic mobility of the solute is determined from the observed migration time (t_M), after correction for the electroosmotic flow and viscosity:

$$\mu_A = \frac{L_d}{E}\left(\frac{1}{t_{M,A}} - \frac{1}{t_{eo}}\right)\frac{\eta}{\eta_0} \quad (1)$$

where L_d is the capillary length to the detector (0.040 m), E is the electric field strength (V/m), $t_{M,A}$ is the observed migration time for the solute in seconds, t_{eo} is the migration time observed for the neutral mesityl oxide, and η/η_0 is the viscosity of the buffer containing cyclodextrin, relative to the viscosity of the buffer alone.

The physicochemical parameters (K, μ_{ACD}, μ_A) were determined by fitting Eq. 3 to the data presented in Fig. 1 using the non-linear curve fitting function of SlideWrite *Plus* (Version 2.0 for Windows, Advanced Graphics Software, Carlsbad, CA, USA). This function uses the iterative Levenberg-Marquardt algorithm which yields parameters based on the minimization of the sum of the squared deviations.

3. Results and discussion

Cyclodextrins are cyclic oligosaccharides consisting of several D(+)-glycopyranose units joined via α-1,4 linkages [19–21]. The three-dimensional conformation of cyclodextrins displays an open cavity. The outside of this cavity is hydrophilic, making cyclodextrins soluble in aqueous solution. The inside of the cavity is hydrophobic in nature. Molecules of the appropriate size (A) can enter the cavity to form an inclusion complex with cyclodextrins (CD):

$$A + CD \overset{K_{ACD}}{\rightleftharpoons} ACD \quad (2)$$

Formation of the inclusion complex is facile on the electrophoretic time scale. Thus the apparent mean

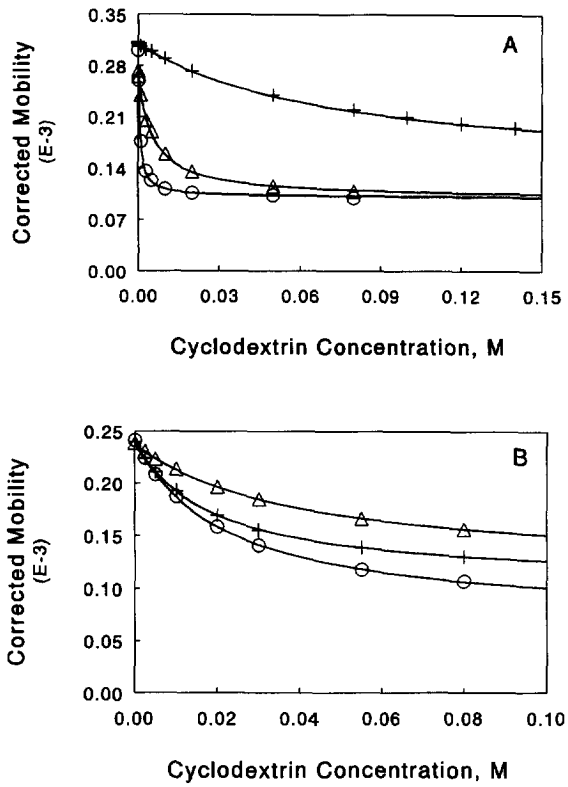

Fig. 1. Effect of α-cyclodextrin concentration on the apparent electrophoretic mobilities of structure isomers. Plot A, *ortho* (+), *meta* (△) and *para* (○) nitrophenolates. Experimental conditions: Buffer, aqueous 10 m*M* phosphate (pH 11.1); temperature, 25°C; voltage, 15 kV; capillary, 75 μm internal diameter, 47 cm long (40 cm to detector); detection, 254 nm. Plot B, 2- (+), 3- (△) and 4- (○) phenyl butyrates. Experimental conditions: Buffer, aqueous 20 m*M* Tris, pH 7.10; temperature, 25°C; voltage, 15 kV; capillary, 75 μm internal diameter, 47 cm long (40 cm to detector); detection, 214 nm. The curves shown are the "best fit" achieved using non-linear curve fitting to Eq. (3). The resultant regression parameters are presented in Table 1.

mobility of a molecule A ($\bar{\mu}_A$) is the weighted average of the intrinsic mobility of the ion (μ_A) and the mobility of the ion–CD complex (μ_{ACD}) [16,18]:

$$\bar{\mu}_A = \alpha_A \mu_A + \alpha_{ACD} \mu_{ACD} = \frac{\mu_A + \mu_{ACD} K_{ACD}[CD]}{1 + K_{ACD}[CD]} \quad (3)$$

where α_A is the concentration fraction of the molecule in the uncomplexed form, α_{ACD} is the concentration fraction of the molecule complexed by cyclodextrin and K_{ACD} is the formation constant of the inclusion complex. Expressions have been derived for the more complex case where the solute molecule is simultaneously involved in an acid dissociation equilibrium [13–15].

In these studies, *o*-, *m*- and *p*-nitrophenol are used as model positional isomers and 2-, 3- and 4-phenyl butyrate are used as model structural isomers. Unfortunately, the 2-phenyl butyric acid and 3-phenyl butyric acid also possess chiral centers. Previous studies have demonstrated that only protonated carboxylic acids undergo enantioselective interactions with cyclodextrins [13,15]. Also, the presence of secondary equilibria (e.g., acid dissociation) complicates the observed behavior [13–15]. Thus, all separations were conducted under conditions for which only the ionized form of the solute is present (i.e., pH greater than 2 pH units above the pK_a of the weakest acid in each set: 4-phenyl butyric acid, pK_a 4.76 [22]; *m*-nitrophenol, $pK_a = 8.0$ [23]).

Under these conditions, Eq. 3 is appropriate to describe the effects observed herein. Thus, the mobility of a solute in the presence of cyclodextrins is a function of its intrinsic mobility, the mobility of its cyclodextrin complex, the formation constant of the inclusion complex and the cyclodextrin concentration. Fig. 1 shows the effect of α-cyclodextrin complexation on the mobility of the isomers of nitrophenolate (plot A) and phenyl butyrate (plot B), respectively. In both cases, the mobility decreases asymptotically from the intrinsic mobility of the solute (μ_A) to the mobility of the inclusion complex (μ_{ACD}) due to formation of the inclusion complex. The curves shown in Fig. 1 are the best fit of the data to Eq. 3, using the free cyclodextrin concentration as per Penn et al. [24]. The coefficient of determination (r^2) was greater than 0.996 for the nitrophenolates, and greater than 0.999 for the phenyl butyrates. The resultant estimates of the formation constant of the inclusion complex (K_{ACD}), the intrinsic mobility of the solute (μ_A) and the mobility of the complex (μ_{ACD}) are given in Table 1.

The stability constants measured in this work for the inclusion complexes between the nitrophenolates and α-cyclodextrin are in excellent agreement with the literature. The values obtained agree with the literature values within the 95% confidence level. The mobilities of the nitrophenolates and their

Table 1
Formation constants, solute electrophoretic mobility and inclusion complex mobilities of solutes with cyclodextrins[a]

Solute		K (M^{-1})	μ_A (10^{-8} m^2 V^{-1} S^{-1})	μ_{ACD} (10^{-8} m^2 V^{-1} S^{-1})
o-Nitrophenol[b]	this work	14±2	3.11±0.01	1.37±0.10
	[24][c]	11±0.2	–	0.99±0.03
m-Nitrophenol[b]	this work	190±10	2.71±0.02	1.00±0.02
	[24][c]	224±11[d]	2.82	0.93±0.01
	[25][e]	202±3	–	–
p-Nitrophenol[b]	this work	1680±60	3.00±0.01	1.01±0.01
	[24][c]	1830±63[d]	2.94	0.93±0.01
	[25][e]	1800±300	–	–
2-Phenyl butyric acid[f]		54±1	2.40±0.01	1.05±0.01
3-Phenyl butyric acid[f]		27±1	2.38±0.01	1.18±0.02
4-Phenyl butyric acid[f]		48±1	2.42±0.01	0.71±0.01

[a]The physicochemical parameters were determined using non-linear fitting of the data in Fig. 1 to Eq. 3. The uncertainties shown are the standard deviations associated with the regression values.
[b]Buffer, aqueous 10 m*M* phosphate (pH 11.1); temperature, 25°C; voltage, 15 kV; capillary, 75 μm internal diameter, 47 cm long (40 cm to detector); detection, 254 nm.
[c]Determined by capillary electrophoresis: buffer, aqueous 50 m*M* phosphate (pH 11.1); temperature, 25°C; voltage, 15 kV; capillary, 50 μm internal diameter, 57 cm long (50 cm to detector); detection, 230 nm.
[d]Corrected to 25°C from the measured (based on mobility of benzoate ion) temperature of 27.8°C.
[e]Measured calorimetrically at 298.15 K.
[f]Buffer, aqueous 20 m*M* Tris, pH 7.10; temperature, 25°C; voltage, 15 kV; capillary, 75 μm internal diameter, 47 cm long (40 cm to detector); detection, 214 nm.

complexes are also consistent with the values reported in the literature, given the lower ionic strength used in this study. The mobilities of the three nitrophenolates (μ_A) are statistically different.

The mobilities of the *m*- and *p*-nitrophenolate inclusion complexes (μ_{ACD}) are statistically equivalent to one another, despite the stability of their complexes differing by almost an order of magnitude. Furthermore, the *o*-nitrophenolate complex is distinctly (based on the 95% confidence limits) faster than the other isomers, despite its very weak formation constant. More surprisingly, the observed complex mobilities also do not correlate with the "quality of fit" of the complex, as depicted in Fig. 2. This figure is an adaptation of Figure 2 from reference [26]. It shows representations of the inclusion complexes of the nitrophenols with α-cyclodextrin, based on X-ray crystal structures of the *meta*- [27] and *para*-nitrophenol [28] inclusion complexes and on a computer-projected model of the *ortho*-nitrophenol complex based on the X-ray structures of the individual solute and cyclodextrin [26]. The complexes are shown in side view with the cyclodextrin represented as a simple cone to better illustrate the degree of penetration. The *p*-nitrophenol (Fig. 2A) penetrates completely into the cyclodextrin cavity with the nitro-group in a position to possibly interact

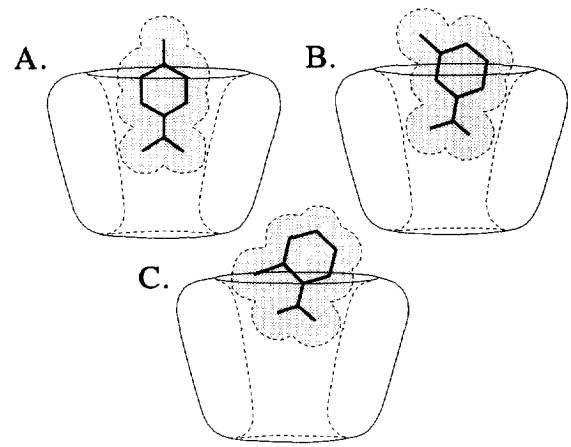

Fig. 2. Computer generated representations of the inclusion complexes between α-cyclodextrin and (a) *p*-nitrophenol, (b) *m*-nitrophenol and (c) *o*-nitrophenol, based on indices determined by X-ray crystallography. The complexes are shown in side view with the cyclodextrin represented as a simple cone to better illustrate the degree of penetration. Adapted from Fig. 2 of reference [26].

with the 6-hydroxyl groups on the lower rim of the α-cyclodextrin. In contrast, the *m*-nitrophenol (Fig. 2B) penetrates only part way into the cavity. Finally, the *o*-nitrophenol hardly penetrates the cavity at all (Fig. 2C), as the radius of the molecule now exceeds the diameter of the opening of the cavity. Based on the radii of the complexes, one would predict complex mobilities of *para*>*meta*>*ortho*. This is almost the opposite of the behavior observed for the complexes. The conclusions drawn from this are that the mobilities of the inclusion complexes of geometric isomers (μ_{ACD}) may differ and that there are no correlations that allow a priori prediction of these mobilities. This has ramifications for the optimization of separations of isomers using cyclodextrin-mediated CZE, as will discussed below.

Fig. 1B shows the experimentally observed mobilities for 2-, 3- and 4-phenyl butyrate for a range of α-cyclodextrin concentrations. The fundamental parameters derived from non-linear fitting of Eq. 3 to this data are presented in Table 1. No literature values for these parameters were available. Nevertheless, we are confident in the values reported in Table 1 for a number of reasons. Firstly, the methodology has been validated based on the nitrophenolates. Secondly, the isomers (2- and 3-phenyl butryate) that possess chiral centers each yield only a single peak. Furthermore, the efficiency of these peaks is statistically equivalent to that of the achiral 4-phenyl butyrate, indicating that no appreciable enantiomer-dependent mobility is occurring. Finally, the coefficients of determination were excellent for each of the phenyl butyrates.

Once again, the complex mobilities for the phenyl butyrates, like the nitrophenolates, differ from one another. This difference in the complex mobilities is significant since all models of cyclodextrin-mediated enantiomeric separation assume that the mobilities of the complexes are equivalent (i.e., $\mu_{ACD}=\mu_{BCD}$). This assumption has been confirmed for the enantiomeric separations of fenoprofen and ibuprofen using β-cyclodextrin [13], of naproxen using hydroxypropyl β-cyclodextrin [15] and the cationic homatropine with β-cyclodextrin [14]. However, the inclusion complex mobilities are not equal when dealing with positional or structural isomers, as was shown above. Thus, modifications to the equations derived to describe cyclodextrin-mediated CZE of enantiomers are necessary to describe the separation of positional and structural isomers.

The cyclodextrin-induced separation can be described by the difference in the apparent mobility between solutes A and B in the presence of cyclodextrins:

$$\Delta\mu = \frac{\mu_A + \mu_{ACD}K_{ACD}[CD]}{1 + K_{ACD}[CD]} - \frac{\mu_B + \mu_{BCD}K_{BCD}[CD]}{1 + K_{BCD}[CD]} \quad (4)$$

Typically, the addition of cyclodextrins is performed only if the intrinsic mobilities of the solutes are identical. Therefore it can be assumed that μ_A equals μ_B. Wren and Rowe [16] used this assumption and the assumption that the complex mobilities are equal ($\mu_{ACD}=\mu_{BCD}$), to derive the following equation to describe enantiomeric separations:

$$\Delta\mu = \frac{(\mu_A - \mu_{ACD})(K_{BCD} - K_{ACD})}{1 + (K_{ACD} + K_{BCD})[CD] + K_{ACD}K_{BCD}[CD]^2} \cdot [CD] \quad (5)$$

Wren and Rowe [16] demonstrated that optimum separation was achieved at the cyclodextrin concentration given by:

$$[CD]_{optimum} = \frac{1}{\sqrt{K_{ACD}K_{BCD}}} \quad (6)$$

However, since the complex mobilities are not equal ($\mu_{ACD}\neq\mu_{BCD}$) in the separation of positional and structural isomers, the assumptions implicit in Eqs. 5 and 6 are not valid. Therefore, to describe the separation of positional and structural isomers by cyclodextrin-mediated CZE, the more general Eq. 4 was used. Fig. 3 shows plots of the observed mobility difference ($\Delta\mu$) versus the α-cyclodextrin concentration for the nitrophenolates (plot A) and the phenyl butyrates (plot B). The symbols are the experimentally determined points and the curves are generated with Eq. 4 using the parameters in Table 1. In both figures, the fit between the experimental data and Eq. 4 is excellent.

The arrows in Fig. 3 indicate the optimum cyclodextrin concentration predicted by Eq. 6. For both the positional (Fig. 3A) and structural (Fig. 3B) isomers, Eq. 6 provides a reasonable prediction of

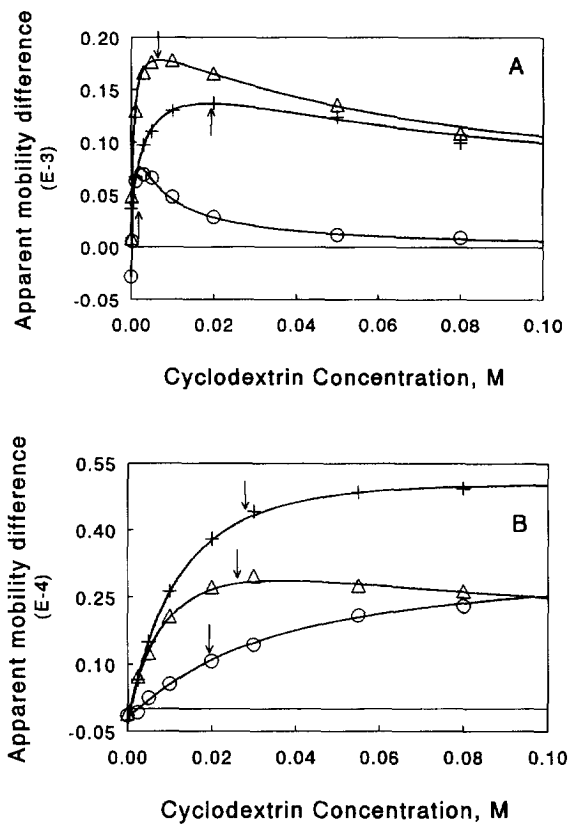

Fig. 3. Effect of α-cyclodextrin concentration on the apparent differences in mobility of (A) nitrophenolates and (B) phenyl butyrates. Conditions are as in Fig. 1. Curves are generated using Eq. 4. Arrows indicate the optimum α-cyclodextrin concentration predicted by Eq. 6. Plot A, *ortho* vs. *meta* (+); *ortho* vs. *para* (△); *meta* vs. *para* (○). Plot B, 2 vs. 3 (△); 2 vs. 4 (○); 3 *vs.* 4 (+).

the optimum α-cyclodextrin concentration. Only the separation of the 2- and 4-phenyl butyrates displays an optimum significantly different from that predicted by Eq. 6. Therefore, only in the case where the formation constants for the isomers are almost equal does the complex mobilities affect the optimum conditions. Thus, while the underlying assumptions upon which Wren and Rowe derived Eqs. 5 and 6 are not necessarily valid in the case of positional and structural isomers, the expressions nonetheless provide a reasonable prediction of the optimum cyclodextrin concentration.

4. Conclusions

This work demonstrates that optimization of isomer separations by cyclodextrin-mediated CZE can depend upon the mobility of the inclusion complexes, in addition to the formation constants of the inclusion complexes. Unfortunately, no correlation with solute mobility, formation constant or "quality of fit" was found that would allow a priori prediction of the complex mobility.

Nonetheless, optimum cyclodextrin concentrations predicted on the basis of the formation constants alone [16] were reasonable in almost all cases. Only when the formation constants for the complexes of the two isomers were approximately equal did the complex mobilities significantly alter the optimal conditions. Thus, even though models for cyclodextrin-mediated CZE, such as that of Wren and Rowe [16], were developed specifically for enantiomeric separations, their predictions provide guidance for the optimization of the separations of other types of solutes as well.

Acknowledgments

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