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Journal of Chromatography A, 768 (1997) 29–38

JOURNAL OF CHROMATOGRAPHY A

Systematic approach to links between separations in capillary electrophoresis and liquid chromatography IV. Application of binding constant–retention factor relationship to the separation of 2-, 3- and 4-methylbenzoate anions using β-cyclodextrin as selector

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Abstract

Quantitative links between binding constants determined in CE and retention factors in HPLC for 1:1 analyte–selector complexes, developed in a previous paper and applied to separations of cationic tioconazole enantiomers with β-cyclodextrin (β-CD), have been extended to the separation of anionic positional isomers. In this paper we apply our unified theory to the separation of anionic methylbenzoate anions (MB^-), again using β-CD as selector. There is only partial resolution for all of the three isomeric methylbenzoates without β-CD present, either in the CE background electrolyte or immobilised in the HPLC experiments. Using the same buffer as in CE (ammonium phosphate pH 7.0), and calculating the effective concentration of selector from the amount of β-CD divided by the volume of the mobile phase in the column, HPLC is shown to give effective binding constants, K', which are in excellent agreement with binding constants, K, determined from the variation of CE mobility with β-CD concentration. $K'=11$, 41, 87 M^{-1} and $K=13.0$, 45.5, 85.3 M^{-1} for 2-, 3-, 4-MB^-, respectively. This demonstrates the quality of our unified treatment of separations based on binding equilibria, and suggests the potential of method development in CE and information transfer to HPLC or vice versa. Quantitative determination of K in CE also generates mobilities of free analyte and analyte–selector complexes, which after correction for non-ideality give radii and diffusion coefficients of the MB^- and MB^-:β-CD ions. This in turn allows a quantitative treatment of variance contributions from diffusion to CE peak widths. Good agreement is found between calculated and observed peak widths when these are measured with the fastest possible data acquisition parameters. ©1997 Elsevier Science B.V.

Keywords: Retention factors; Binding constants; Unified separation theory; Geometrical isomers; Diffusion coefficients; Methylbenzoate; Cyclodextrins

1. Introduction

In recent publications a quantitative link has been established between binding constants (K) determined in capillary electrophoresis (CE) and retention factors in high-performance liquid chromatography (HPLC) where the same selector is employed in both separation modes [1,2]. The previous paper in this series introduced this treatment and exemplified it with β-cyclodextrin (β-CD) as a mobile phase

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PII S0021-9673(97)00144-1

additive in CE and as an immobilised selector in HPLC for the chiral separation of tioconazole [1], while Ahmed et al. used human serum albumin (HSA) for the separation of benzoin in HPLC and affinity CE [2].

Methods for separation in CE are generally easier to develop than in HPLC, where a greater number of variables require optimising. In CE, chiral separations are normally achieved using mobile phase additives that complex with the charged enantiomeric species forming a dynamic equilibrium between free analyte and diastereomeric complex. The most frequently used chiral selectors for CE are cyclodextrins (both native and derivatised) [3–8]; these have a number of advantages including stability over a wide pH range, high solubility in polar and non-polar solvents [9] and cost effectiveness in comparison to other selectors and techniques [10]. Cyclodextrins have also been used in CE to separate positional isomers [11–14].

Chiral HPLC is well established for the separation of enantiomers through diastereomeric complexation and is currently the most common technique for enantioseparations; the use of immobilised cyclodextrins is exemplified by references [15–17]. Few papers have been reported using immobilised cyclodextrins for the separation of positional isomers, presumably because alternative methods are available on achiral bonded phases. Abidi and Mounts [18] however described the use of an immobilised γ-cyclodextrin phase for the separation of tocopherols and methylated tocols in reversed-phase HPLC.

The present paper describes the use of β-cyclodextrin as a selector for the separation of the geometric isomers 2-, 3- and 4-methylbenzoate anions by their differential inclusion into the cyclodextrin cavity. The aim is to compare the migration/elution order involved in the two separation modes and to validate further our proposed relationship between binding constants relevant to CE using cyclodextrins and retention factors in HPLC using the same immobilised cyclodextrin as selector.

2. Theory

In our previous publication the relationship between binding constants (K) in CE and retention factors (k') in HPLC was established [1]. In CE, binding constants may be calculated by fitting data for analyte mobility over a range of selector concentrations using Eq. (1) [8,19]

$$\mu = \frac{(\mu_0 - \mu_\infty)}{(1 + KC)} + \mu_\infty \quad (1)$$

where μ, μ_0 and μ_∞ are the observed analyte mobility, mobility at zero selector concentration and mobility at infinite selector concentration, respectively and C is the concentration of *free* selector. Eq. (1) gives μ as the average mobility with contributions from μ_0 and μ_∞ weighted according to the relative amounts of free and bound analyte.

In HPLC we define the effective binding constant, K', as

$$K' = \frac{c_{SA}}{c_A c_S} = \frac{(n_{SA}/V)}{(n_A/V)c_S} = \frac{n_{SA}}{n_A c_S} \quad (2)$$

where c_{SA}, c_A and c_S are the concentrations of selector–analyte complex, free analyte and free selector, respectively, n_{SA}, n_A are the number of moles of selector–analyte complex and analyte, respectively and V the volume of the system. Since by definition the retention factor, $k' = n_{SA}/n_A$

$$K' = \frac{k'}{c_S} \quad (3)$$

The *effective concentration of selector*, c_S, may be calculated by dividing the total number of moles of selector, n_S, present in the column by the total solution phase volume of the column, V. For cyclodextrins on typical silica supports, values for c_s are calculated to be in the range of $(1–10)\cdot 10^{-2}$ M [1], the lower and upper bounds corresponding to 10% and 100% surface coverage, respectively. From this it follows that

$$K'\,(M^{-1}) \sim (10 - 100)k' \quad (4)$$

For the ideal case where the support and tether for the selector have no effect on the binding constant, $K = K'$, and there should be a quantitative link between CE and HPLC with $K = k'/c_S$. Previous investigations to analyse the effect of the tether found that it played little part in retention and selectivity [20].

Table 1
Capacity factor ranges for given binding constant values found in free solution

$K\ (M^{-1})$	k' range
10^4	100–1000
10^3	10–100
10^2	1–10
10^1	0.1–1

From Eq. (4), a value of $K = 10^2\ M^{-1}$ corresponds to k' in the range 1–10. As this is the desired working range for LC, a binding constant in CE of $\sim 10^2\ M^{-1}$ should allow direct transfer of conditions from CE to LC. Table 1 shows the capacity factor ranges in HPLC for given binding constant values found in free solution CE.

3. Experimental

3.1. Materials

2-, 3- and 4-methylbenzoic acids were purchased from Aldrich (Gillingham, UK) as was the neutral marker, mesityl oxide. Native β-cyclodextrin (β-CD) was a gift from Wacker Chemicals (Halifax, UK). AR grade diammonium hydrogenphosphate, disodium hydrogenphosphate and phosphoric acid were all purchased from Fisons (Loughborough, UK). In both the CE and HPLC investigations, buffers consisting of 25 m*M* diammonium hydrogenphosphate or disodium hydrogenphosphate titrated to pH 7.0 with concentrated phosphoric acid were used. All CE background electrolytes (BGEs) and HPLC mobile phases were filtered prior to use through 0.2 μm filters.

3.2. Methods

All CE experiments were carried out on an automated instrument (Beckman PACE 2100). The capillary used (Composite Metals, Hallow, UK) was 47 cm in length (40 cm to the detector) × 50 μm I.D. The capillary was thermostatted at 25°C. The following conditions applied for the analysis; voltage 30 kV; UV detection at 230 nm; 2 s pressure injection (2 nl) of 0.1 m*M* analyte dissolved in the BGE. Between injections the capillary was rinsed for 2 min with 0.1 *M* NaOH followed by a 5 min rinse with the run buffer. Unless specified, all CE data were collected using Beckman System Gold with 1.0 s rise time and 5 Hz collection rate.

Relative viscosities were measured using a capillary viscometer (Townson and Mercer, Croydon, UK) thermostatted at 25°C. These values were subsequently fitted to a concentration power series [21] and from this the corrected enantiomer mobilities were calculated using the equation [8,19]

$$\mu = \mu_{obs} \cdot \frac{\eta_c}{\eta_0} \tag{5}$$

where μ is the corrected analyte mobility, μ_{obs} the observed mobility, η_c and η_0 are the viscosities of the solutions at cyclodextrin concentration c and 0, respectively.

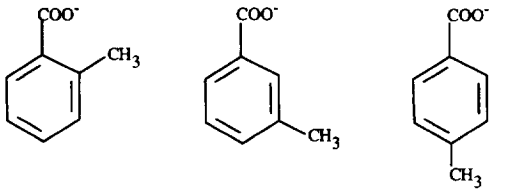
Components in the HPLC system used were; pump (ACS Model 352); injector (Rheodyne 7152) with 20 μl loop; variable wavelength UV detector (ACS 750/12) set at 230 nm; integrator (Trivector Trio). A Cyclobond I 2000 β-CD column (250 mm × 4.6 mm I.D. Astec, Whippany, NJ, USA) was used and thermostatted at 25°C. A test mix was produced with each methylbenzoic acid at a concentration of 0.1 m*M* in water. A 0.01 m*M* test-mix which was used in a number of the HPLC investigations was produced by diluting the 0.1 m*M* mix tenfold. All CE experiments were carried out in triplicate and all HPLC experiments at least in duplicate.

4. Results and discussion

4.1. CE studies

4.1.1. Migration order

Structures of the methylbenzoate anions (MB^-) are given in Fig. 1. An electropherogram of a mixture of the isomers with no cyclodextrin in the pH 7.0 BGE is shown in Fig. 2a. Being negatively charged, the analytes migrate after the electroosmotic flow (EOF) marker. All isomers are seen to have similar migration times, and 2- and 3-methylbenzoates comigrate. The effect of addition of β-CD at a concentration of 16 m*M*, its solubility limit in water, is shown in Fig. 2b. The EOF shifts to slightly longer time (due to the increase in viscosity of the BGE),



2-MB⁻ 3-MB⁻ 4-MB⁻

Fig. 1. Geometric isomers of methylbenzoate anions.

the migration times of 4- and 3-MB⁻ decrease, and very good separation of all three analytes is observed. This illustrates how cyclodextrins can be used to separate geometric isomers. The migration order, 4->3->2-MB⁻, was elucidated by spiking samples with the single isomers and follows the order of the binding strengths of the CD–MB⁻ complexes. The most strongly bound isomer, having the lowest average mobility counter to the EOF, migrates closest to the neutral marker. An alternative way to separate the comigrating 2- and 3-isomers in CE would be to use differential binding of protons, and adjusting the pH to fall between the pK_a values for the two isomers [22]. Our aim however in this paper is to link CE and HPLC separations, by focusing on the use of the β-CD selector either as a BGE additive (in CE) or a bonded phase (in HPLC).

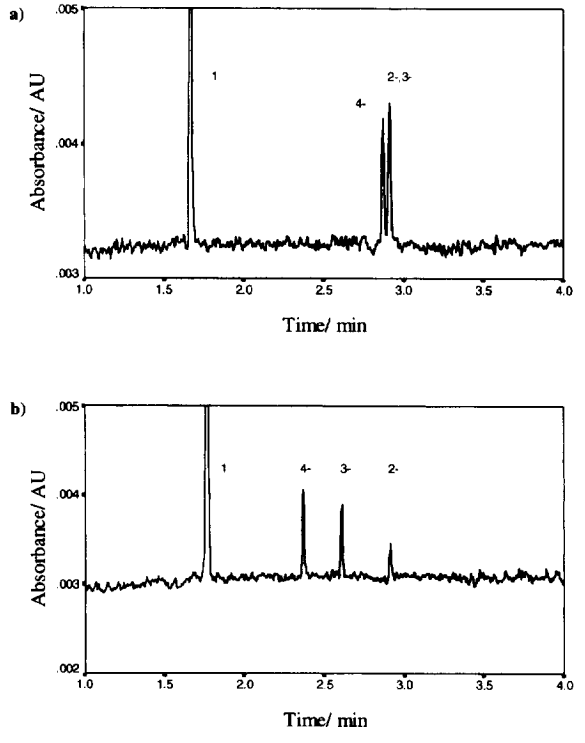


Fig. 2. Separation of methylbenzoates at (a) 0 m*M* β-CD and (b) 16 m*M* β-CD. Conditions: Capillary 47 cm (40 cm to detector) × 50 μm I.D. fused-silica thermostatted at 25°C; buffer 25 m*M* $(NH_4)_2HPO_4$ titrated to pH 7.0 with concentrated H_3PO_4; voltage 30 kV; absorbance 230 nm; 2 s (2 nl) pressure injection of 0.1 m*M* test mix of each component. Peak identification: (1) EOF (mesityl oxide); (2) 2-methylbenzoate; (3) 3-methylbenzoate; (4) 4-methylbenzoate.

4.1.2. Binding constants

Observed electrophoretic mobilities, μ_{obs}, were calculated from migration times of the analytes and neutral marker and allowance made for viscosity variation (Eq. (5)) to give mobilities, μ, corrected to aqueous BGE conditions [8,19]. Fig. 3 shows the variation in μ for the three isomers as a function of β-CD concentration. Curves fitted through the data points are non-linear least-squares fits using Eq. (1) [19]. For 3- and 4-MB⁻, both variables μ_∞ and K were extracted from the data. For the weakly-binding isomer, 2-MB⁻, data points are restricted to ~15% of the maximum mobility difference between free and bound forms, and cover too small a proportion of the binding range to allow both variables to be independently extracted with confidence. In this case the average of μ_∞ values for 3- and 4-MB⁻, with a

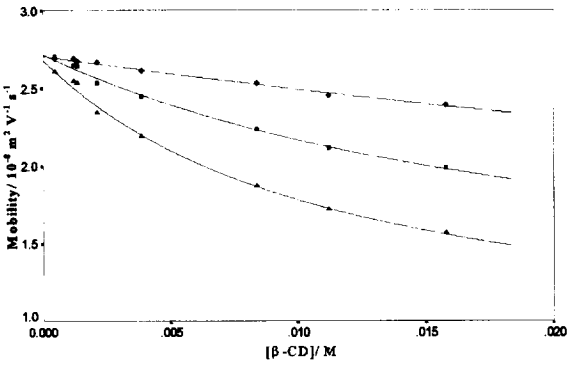


Fig. 3. Fitting of mobility data of methylbenzoate isomers over the β-cyclodextrin concentration range of 0–16 m*M* in ammonium phosphate buffer pH 7.0. Curve identification; 2-methylbenzoate (♦), 3-methylbenzoate (■) and 4-methylbenzoate (▲). Binding constants shown in Table 2. Experimental conditions described in Section 3.2.

generous limit of uncertainty, was assumed for μ_∞ for 2-MB^- and used to calculate K. This assumption is based on the quantitative link between μ and hydrodynamic radius [23]. Since with these relatively small analytes the radii of the host–guest CD–MB^- complexes are expected to be principally dependent on the hydrodynamic radius of the host CD species, μ_∞ values should be similar for all three isomers.

It is known that sodium and potassium phosphate buffers have a detrimental effect on the stability of Cyclobond columns [24]. Furthermore, these columns become unstable outside the pH range 3.0–7.0. For these reasons the initial buffer, 25 mM $(NH_4)_2HPO_4$ titrated to pH 7.0, was chosen to allow for direct transfer of conditions to the HPLC experiments. In order to check whether the choice of buffer cation had any effect on binding constant, the buffer counter-ion was changed from ammonium to sodium ion. The result of the analysis using both buffers is shown in Table 2.

Quantitative values for K in Table 2 confirm the qualitative deductions about the binding strengths, in the order 4->3->2-MB^-.

When comparing data for NH_4^+ and Na^+ in Table 2, K is seen to be independent of counter-ion for the 2- and 3-isomers. There is an apparent difference for the 4-isomer, but this could be attributable to differences in the μ_∞ values. When μ_∞ is constrained to be the same, results for K are within each other's 95% confidence limits. Overall, it can be deduced that the change of counter-ion from NH_4^+ to Na^+ has no significant effect on the binding constant. This is consistent with the cation playing no role in binding; similar results have been obtained in studies of fluoxetine and norfluoxetine binding to β-cyclodextrin [25].

4.1.3. Mobilities

Mobilities may be correlated with radii of the ions and also used to calculate diffusion coefficients needed for determination of peak variance. In order to calculate these quantities, all mobilities must be corrected to zero ionic strength. This correction may be made using Eq. (6) below [23]

$$\mu = \mu^0 - \frac{(0.229\mu^0 + 3.12 \times 10^{-8})I^{1/2}}{1 + 3.28aI^{1/2}} \quad (6)$$

where μ^0 is the limiting or absolute mobility, I the ionic strength, a the sum of the ionic radii of the analyte ion and counter-ion (nm); the equation with the numerical value shown is applicable to univalent ions in aqueous solution at 298 K.

For the MB^- ions, Table 2 shows that there is a small but significant change in μ_0 on changing the counter-ion. Both solutions have the same ionic strength, $I = 0.064$ mol kg^{-1}. The smaller values of μ_0 in NH_4^+ solutions than Na^+ solutions are attributable to the effects of counter-ion size in Eq. (6); the correction factor scales with a term inversely dependent on $I^{1/2}$ multiplied by the sum of the analyte and counter-ion radii. The centre of the larger Na^+ ion (radius 0.18 nm) cannot get as close to the MB^- ion as in the case of the NH_4^+ ion (radius 0.13 nm). Radii of the solvated ions are calculated from limiting ionic conductivities [26,27] or limiting mobilities [28,23]. For the methylbenzoates, mobility at zero

Table 2
Binding parameters and mobilities at 25°C for methylbenzoate isomers to β-CD

Buffer cation		Isomer		
		2-	3-	4-
(a) NH_4^+	K (M^{-1})	13.0±2.0	45.5±1.2	85.3±2.5
	μ_0 (10^{-8} m^2 V^{-1} s^{-1})	−2.71	−2.72	−2.68
	μ_∞ (10^{-8} m^2 V^{-1} s^{-1})	(−0.83±0.20)	−0.94±0.03	−0.71±0.03
(b) Na^-	K (M^{-1})	13.3±2.0	48.2±0.6	100.3±1.0
	μ_0 (10^{-8} m^2 V^{-1} s^{-1})	−2.75	−2.75	−2.72
	μ_∞ (10^{-8} m^2 V^{-1} s^{-1})	(−0.90±0.20)	−0.97±0.01	−0.82±0.01

Buffer: (a) 25 mM diammonium hydrogen phosphate titrated to pH 7.0 with concentrated H_3PO_4; (b) 25 mM disodium hydrogen phosphate adjusted as in (a) to pH 7.0.

ionic strength, μ_0^0, was calculated from μ_0 data in Table 2 using Eq. (6). Both μ_0 values of $-2.75 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} with Na^+ as the counter-ion and $-2.72 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} with NH_4^+ counter-ion correct to the same $\mu_0^0 = -3.46 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} for the 3-isomer. Similarly for the 4-isomer, data with both counter-ions gives $\mu_0^0 = -3.42 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}. Mobilities at zero ionic strength for the β-CD–MB^- complexes, μ_∞^0 are calculated to be -1.3, -1.4 and $-1.2 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} for the 2-, 3- and 4-isomers, respectively. It should be noted that the corrections to zero ionic strength are more significant the larger the ion. For the free MB^- ions, $\mu_0^0 : \mu_0$ equals 1.27, whereas for the β-CD–MB^- complexes $\mu_\infty^0 : \mu_\infty = 1.6$.

The hydrodynamic radius of the complex may be determined from the Stokes law expression

$$r = \frac{ze}{6\pi\eta\mu^0} \quad (7)$$

where z is the charge of the ion, e the fundamental electronic charge and all other symbols have been defined previously. For aqueous solutions at 298 K, Eq. (7) gives r (nm) $= 9.54 \cdot 10^{-9}\, z/\mu^0$. Applying Eq. (7) to the complex suggests $r \sim 0.7$–0.8 nm, in good agreement with the dimensions of a β-CD molecule, with height 0.7 nm and external radius 0.8 nm [9].

4.1.4. Contributions to peak variance

In our previous paper [1], the effects of various contributions to observed peak broadening for the enantiomers of tioconazole were discussed. After diffusion, injection and detection, which should be the main contributors to variance, had been taken into account, a significant proportion of the variance was still unaccounted for. It was reasoned that this additional band broadening with these positively-charged species was due to analyte–wall interaction.

The methylbenzoates, with pK_a values of 3.91, 4.27 and 4.37 for the 2-, 3- and 4-isomers, respectively [29] are all negatively charged under the experimental conditions. Any pK_a shifts due to inclusion into the cyclodextrin cavity [9] are unlikely to be great enough to alter the charge state in a pH 7.0 buffer. Thus no interaction between the capillary wall and analytes should occur.

Initial calculations from the electropherograms of Fig. 2 suggested that, for these fast separations (~3 min), diffusion coupled with injection and detection variance accounted for no more than 50% of the observed peak broadening. However, this was found to be in part an artefact of the rise time of the integrator. The normal CE rise time of 1 s, the default setting, was insufficiently fast to measure the true peak widths of the analytes. The experiments were subsequently repeated with the fastest data acquisition parameters selectable, 0.1 s rise time and 20 Hz data collection rate. A second factor which needs to be taken into account is the time between injection and the start of migration. For all analytes, this was measured to be 34 s. This time is made up of contributions from (i) rotations of the sample carousel, (ii) raising and lowering of the vials, (iii) injection of a 2 s buffer plug after the sample (to prevent analyte loss upon application of the electric field [30]) and (iv) half the voltage ramp time [31].

Results of calculations of contributions to peak variance are shown in Table 3. Equations used are from Reference [32]. Key physical parameters and variance contributions are as follows: $\bar{D}$ is the average diffusion coefficient of the analyte weighted according to the proportions of the free and complexed forms, calculated from

$$\bar{D} = \frac{\mu^0 kT}{ze} \quad (8)$$

μ^0 is the analyte mobility corrected to zero ionic

Table 3
Key physical parameters and variance contributions for methylbenzoate isomers with 16 m*M* β-CD in ammonium phosphate buffer pH 7.0

Isomer	t_m (min)	$w_{1/2}$ (s)	D (10^{-10} m^2 s^{-1})	σ^2_{obs}	σ^2_D (10^{-7} m^2)	$\sigma^2_i + \sigma^2_{det}$	$\sigma^2_{calc}/\sigma^2_{obs}$ (×100, %)	N_{obs}
2-	3.566	0.85	8.0	5.3	4.0	1.4	102	$3.0 \cdot 10^5$
3-	3.118	0.77	6.7	4.7	3.1	1.4	97	$3.4 \cdot 10^5$
4-	2.782	0.64	5.4	4.2	2.4	1.4	89	$3.8 \cdot 10^5$

Temperature 25°C.

strength (see Section 4.1.3) using μ_0^0 and μ_∞^0 values in place of μ_0 and μ_∞ in Eq. (1), together with K values from Table 2 and the β-CD concentration 16 mM. k is the Boltzmann constant and T the absolute temperature. σ^2_{obs} is the observed peak variance, σ^2_D the variance due to diffusion, σ^2_i the variance due to injection and σ^2_{det} the detection variance. $\sigma^2_{calc}/\sigma^2_{obs}$ is the fraction of the three principal variance contributors to the observed peak variance and N is the number of theoretical plates. The term for σ^2_D in Table 3 includes a contribution from diffusion of the free analyte during the time (34 s) between injection and the start of migration.

Results give 100 $\sigma^2_{calc}/\sigma^2_{obs}$ of 102, 97 and 89% for the 2-, 3- and 4-isomers, respectively. The key point from these calculations is that the principal variance parameters now account for all of the total observed variance, within the uncertainties of both measurement and theory. Our results show that true peak widths are only detectable with a fast rise time and collection rate. Diffusion and injection contributions dominate the observed variance, and for these anionic species no contribution occurs from wall interactions. This results in very high peak efficiencies.

4.2. HPLC studies

The binding constants of all the analytes determined in both separation buffers in CE are ≤ 100 M^{-1}. As discussed in Section 2, this binding constant range is desirable for direct transfer of conditions to HPLC. Had the binding constant required lowering to bring it into the required range, then organic modifiers such as methanol [8] or acetonitrile [1] could have been used.

4.2.1. Elution order

As mentioned previously, the CE buffer system of 25 mM $(NH_4)_2HPO_4$ was used for direct method transfer to HPLC, and would thus allow direct comparison of results. A typical chromatogram with a 20 μl loading of the 0.01 mM test-mix of the methylbenzoates on the Cyclobond I 2000 column is shown in Fig. 4a. The elution order using this column is, 2-MB^- (least bound)<3-MB^-<4-MB^- (most bound). This is concordant with the binding constant data found from CE.

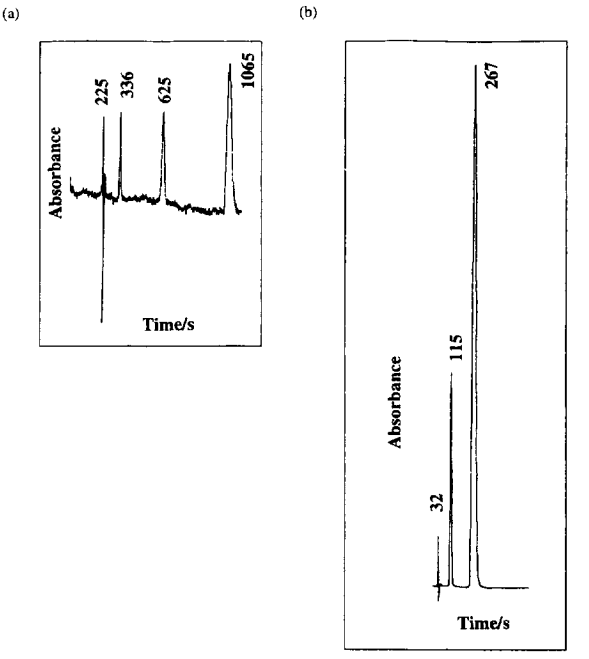


Fig. 4. Chromatogram of methylbenzoate isomers separated on (a) β-cyclodextrin column and (b) C_8 column. Conditions: (a) 25 mM $(NH_4)_2HPO_4$ (pH 7.0) buffer, elution order; 2-methylbenzoate, 3-methylbenzoate, 4-methylbenzoate. Column: 25 cm×4.6 mm I.D. Cyclobond I 2000 (β-cyclodextrin); flow-rate, 0.75 ml min^{-1}; UV detection at 230 nm; injection, 20 μl of test mix (all analytes at 0.01 mM) dissolved in water. (b) as (a) but test mix 0.1 mM in all analytes, flow-rate 1.0 ml min^{-1} and column 5 cm×4.6 mm I.D. Spherisorb C_8. Elution order; 2-methylbenzoate, 3-/4-methylbenzoate (coelution).

To examine if it was possible to easily separate these geometric isomers in a reversed-phase system without cyclodextrins, the separation using a 5 cm× 4.6 mm I.D. Spherisorb C_8 column was also investigated with the ammonium based buffer. The chromatogram is shown in Fig. 4b. Using this column, no selectivity was observed for the 3- and 4-isomers, which coelute in the second peak (identified by sample spiking). As in CE, where comigration was observed when the host–guest selector was not present, this again shows the benefits of using a cyclodextrin selector — in this case as a stationary phase. A possible suggestion for the increased retention of the 3- and 4-isomers relative to 2-MB^- on a C_8 phase is as follows. Referring to the structures in Fig. 1, the hydrophilic carboxylate group in MB^- is likely to be positioned pointing up into the

aqueous phase. This sets the direction of the 3- and 4-Me groups downwards into the hydrophobic C_8 phase, whereas the 2-group lies at the phase boundary where its hydrophobic binding will be weaker.

4.2.2. Effect of LC buffer concentration

The effect on peak separation characteristics of adjusting the buffer concentration over the range 5–100 m*M* is shown in Table 4. The buffer used was 5–100 m*M* $(NH_4)_2HPO_4$ adjusted to pH 7.0 with concentrated H_3PO_4. The analytes were injected at 0.1 m*M* concentration. A_s is the peak asymmetry and α is the selectivity between the peaks noted under "isomer".

The retention factors are independent of buffer concentration, showing that variation in buffer concentration has little effect on the inclusion process in HPLC. This would be consistent with the β-CD interacting with the analytes without any significant electrostatic contributions from the negatively charged silica support. Selectivity (calculated from $\alpha = k'_2/k'_1$, where $k'_2 > k'_1$ [33]) is also independent of buffer concentration. This is similar to the case with tioconazole enantiomer binding to β-CD [1] and is further evidence of the inherent selectivity of the cyclodextrins.

4.2.3. Effect of loading and flow-rate

The effects of variation of loading and flow-rate were investigated using the 25 m*M* $(NH_4)_2HPO_4$ buffer over the range 0.5–1.5 ml min^{-1}, and 0.01 m*M* of each analyte injected in place of the 0.1 m*M* concentrations in Section 4.2.2. The results of this study are shown in Table 5. NH_4^+ was replaced by Na^+ in the column marked with an asterisk.

In terms of loading, comparison of Tables 4 and 5 show that asymmetry factors decrease as the loading is decreased. This is consistent with overloading at

Table 5
Effect of flow-rate on separation parameters

	Isomer	Flow-rate (ml min^{-1})					
		0.50	0.75	1.00	1.00*	1.25	1.50
k'	2-	0.56	0.50	0.45	0.50	0.51	0.50
	3-	2.06	1.79	1.65	1.72	1.74	1.75
	4-	4.49	3.79	3.46	3.59	3.55	3.67
N	2-	9300	6800	5000	5000	3500	4700
	3-	9000	6000	4100	4100	4200	4000
	4-	8200	5200	3500	3500	4500	3700
A_s	2-	1.0	1.5	1.3	1.3	1.0	1.0
	3-	1.3	1.2	1.3	1.4	1.3	1.3
	4-	2.1	1.9	1.5	1.8	1.3	1.6
α	2-,3-	3.68	3.56	3.67	3.44	3.41	3.50
	3-,4-	2.18	2.12	2.10	2.09	2.04	2.10

Analytes injected at 0.01 m*M* concentration. Buffer $(NH_4)_2HPO_4$ adjusted to pH 7.0 with concentrated H_3PO_4 (except * Na_2HPO_4).

Table 4
Effect of buffer concentration on separation parameters

	Isomer	Buffer (m*M*)						
		5	10	15	20	25	50	100
k'	2-	0.75	0.83	0.68	0.71	0.74	0.69	0.71
	3-	2.62	2.91	2.39	2.56	2.51	2.37	2.40
	4-	5.61	6.27	5.13	5.63	5.39	5.15	5.21
N	2-	16 300	7700	6400	6900	6800	6700	6700
	3-	4400	4900	4800	5500	5100	4900	4900
	4-	3400	3400	3900	3700	3600	3100	3500
A_s	2-	1.5	2.0	1.5	1.5	1.3	2.0	1.5
	3-	5.0	2.4	1.8	2.8	2.5	2.8	2.5
	4-	5.5	5.4	4.4	4.8	5.2	5.0	4.5
α	2-,3-	3.49	3.41	3.67	3.67	3.48	3.43	3.48
	3-,4-	2.14	2.13	2.20	2.20	2.16	2.17	2.16

Buffer $(NH_4)_2HPO_4$ adjusted to pH 7.0 with concentrated H_3PO_4. Analytes injected at 0.1 m*M* concentration.

the higher concentration. Retention factors are lower at the lower loading, but there is no significant difference in selectivity.

The effects of flow-rate variation show that efficiencies are highest at lowest flow-rate. Little difference is observed in the retention factors of the three analytes. As in the experiments to determine the effect of the ionic strength on the separation, little change is observed on the selectivity between peaks, which is to be expected if the retention factors do not vary with flow-rate.

Results show that replacing NH_4^+ by Na^+ as buffer counter-ion makes no difference to the separation parameters.

4.3. Linking binding constants in CE and retention factors in LC

The calculation of effective concentration of β-CD in a Cyclobond I 2000 column is as follows. Using elemental analysis and column loading data provided by the suppliers, Astec, $\%C = 6.39$. The spacer arm (3-glycidoxypropyl trimethoxy silane) has a $\%C$ loading of 4.09% and thus, by difference, the $\%C$ loading of β-CD is 2.30%. There is ~2.8 g of silica in the column of which 2.8 g × (2.30%/100%) = 0.064 g is carbon due to the cyclodextrin. The mass of carbon per mol of cyclodextrin (molar mass = 1134 g mol^{-1}) is 504 g mol^{-1} and therefore the number of mol of cyclodextrin is $(0.064/504) = 1.28 \cdot 10^{-4}$ mol. This is the value of n_S^T for this column. The dead time of the column was found to be 3.22 min at a flow-rate of 1.0 ml min^{-1}, corresponding to a dead volume (V) within the column of 3.22 cm^3. From this, the effective concentration of β-CD is $c_s = n_S^T/V = (1.28 \cdot 10^{-4}$ mol/3.22 $cm^3) \times 1000$ cm^3 $dm^{-3} = 0.040$ M.

From Eq. (3), this gives

$$K'(M^{-1}) = 25k' \quad (9)$$

Using k' data from Table 5 (25 mM buffer concentration, 1.00 ml min^{-1} flow-rate and 0.01 mM test-mix injection to avoid overloading effects in HPLC), K' was calculated for all MB^- isomers and both NH_4^+ and Na^+ counter-ions. The correlation between the effective binding constant, K', determined from HPLC and the binding constant K from CE is shown in Table 6. There is excellent agreement, with the ratio K':K within 20% of unity for all three isomers and the two buffer counter-ions.

Table 6
Quantitative link between K and k' for methylbenzoate isomers binding to β-CD in CE and HPLC

Buffer counter-ion	Isomer	k'	K' (M^{-1})	K (M^{-1})	K':K
NH_4^+	2-	0.45	11	13.0	0.8
	3-	1.65	41	45.5	0.9
	4-	3.46	87	85.3	1.0
Na^+	2-	0.50	13	13.3	1.0
	3-	1.72	43	48.2	0.9
	4-	3.59	90	100.3	0.9

5. Conclusions

There is only partial resolution for all of the three isomeric methylbenzoates without cyclodextrins present, either in the CE background electrolyte or immobilised in the HPLC experiments. With the presence of cyclodextrins excellent resolution of the analytes is possible in both separation modes.

In the CE experiments, injection (plus detection) variance and diffusion account for all of the peak width measured with the best possible data acquisition parameters, i.e., a low rise time and high collection rate. Use of the default settings in the CE software gives erroneous results for widths of the very sharp peaks observed with these negatively-charged analytes.

In HPLC, variation of buffer ionic strength has little effect on k' or α. Injection of analyte solution at concentration 0.01 mM of each analyte was found appropriate to avoid overloading and poor values of peak asymmetry.

The elution order and k' values of the analytes in LC correlate with the binding constants from CE. There is excellent agreement between the effective binding constant K', determined from k' in HPLC, and the binding constant K from CE, with the ratio K':K within 20% of unity for all three isomers and the two buffer counter-ions.

This provides strong support for our unified theory linking binding constants in CE and retention factors

in HPLC. Using the same selector, CE may be used as a rapid scouting technique for the development and optimization of an HPLC separation. This could both reduce method development time in HPLC, and also provide quantitative predictions of retention factors. Alternatively, HPLC retention factor data, along with knowledge of effective concentration of the selector from its mass loading in the column, could provide binding constant data for reduced method development time in CE.

Acknowledgments

We would like to thank the EPSRC and Pfizer Central Research for a CASE award to PDF. We also gratefully acknowledge the gift of an Astec Cyclobond I 2000 HPLC column from BAS Technicol.

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