Liquid chromatographic retention behaviour and separation of chlorophenols on a β-cyclodextrin bonded-phase column. Part III. Diaromatic chlorophenols

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This paper is dedicated to Professor Arthur S. Perlin on the occasion of his 67th birthday


The liquid chromatographic retention behaviour of 16 diaromatic phenols and chlorophenols (PCB metabolic analogues) on a β-cyclodextrin bonded-phase column was investigated with respect to mobile-phase composition, pH, temperature, and ionic strength. The mechanistic aspects of retention of these compounds on the β-cyclodextrin column were studied and compared to other reversed-phase columns. Comparison is also made to the liquid chromatographic retention behaviour and separation of monoaromatic chlorophenols on the same column. The size, shape, and spatial geometry of each substrate appears to affect retention and selectivity. Most of the evidence suggests that inclusion complex formation, which involves hydrophobic interactions with the interior of the cavity and/or van der Waals interactions of the chlorine substituents with the backbone of the cavity, provides the physical basis for the resolution of these positional isomers. Under certain chromatographic conditions, however, it appears that, for certain congeners, all or part of the molecule is excluded from the cyclodextrin cavity; in such cases a normal-phase or mixed chromatographic mechanism is postulated based on the interaction of the substrates with the secondary hydroxyls on the periphery of the cyclodextrin moieties. The gradient-elution separation of 14 out of 16 of this type of compound on a single chromatogram is also reported and discussed.

Key words: biphenylols, chlorobiphenylols, biphenyldiols, liquid chromatographic retention behaviour, gradient-elution separation, β-cyclodextrin bonded-phase column.


Faisant varier la composition de la phase mobile, le pH, la température et la force ionique, on a étudié le comportement de seize phénols et chlorophénols diaromatiques (analogues de métabolites de PCB) lors de leur chromatographie liquide de rétention dans une colonne remplie d’une phase liée à la β-cyclodextrine. On a étudié les aspects mécanistiques de la rétention de ces composés sur la colonne de β-cyclodextrine et on a comparé à ceux d’autres colonnes de phase inversee. On a aussi effectué une comparaison avec le comportement et la séparation par chromatographie liquide de chlorophénols monoaromatiques sur la même colonne. Il semble que la rétention et la sélectivité soient affectées par la grosseur, la forme et la géométrie spatiale de chaque substrat. La plupart des données obtenues suggèrent que la formation d’un complexe d’inclusion, qui implique les interactions hydrophobes avec l’intérieur de la cavité et des interactions de van der Waals des substituants chlorés avec le squelette de la cavité, fournit la base physique pour la résolution de ces isomères de position. Toutefois, dans certaines conditions chromatographiques, il semble que, pour certains congénères, l’ensemble des groupements de la molécule soit exclu de la cavité de la cyclodextrine; dans de telles conditions, on envisage un mécanisme chromatographique de phase normale ou mixte qui serait basé sur l’interaction du substrat avec les groupements hydroxyles secondaires à la périphérie de la portion cyclodextrinée. On rapporte aussi la séparation, sur un seul chromatogramme et en faisant appel à un gradient d’éluion, de quatorze des seize composés de ce type et on discute ce résultat.

Mots clés : biphénols, chlorobiphénols, biphénylols, comportement lors de la chromatographie liquide de rétention, séparation par gradient d’éluion, colonne remplie d’une phase liée à la β-cyclodextrine.

Introduction

In two previous publications (1, 2) we discussed the liquid chromatographic (LC) retention behaviour and separation of monoaromatic chlorophenols on a β-cyclodextrin (CD) bonded-phase column. It is the purpose of this paper to extend these investigations to diaromatic chlorophenols of a biphenyl-type structure (see Fig. 1). Stable CD bonded phases were first described in the literature in 1984 (3). These packings consist of chiral CD molecules linked to silica gel via a 6- to 10-atom non-nitrogen-containing spacer arm. Both the linkage and the CD are hydrolytically stable under standard LC conditions. The attachment is such that the CD molecules remain physically intact.

Cyclodextrins are torus-shaped cyclic oligosaccharides formed by the action of Bacillus macerans amylase on starch. These molecules contain six to twelve glucose units bonded through α-(1, 4)-glycosidic linkages. The physical shape of the molecule is that of a truncated cone, with an internal cavity whose dimensions are determined by the number of glucose units. Due to the orientation of the glucose units, there are no hydroxyls on the interior of the cavity thus rendering it hydrophobic.

When using aqueous-organic mobile phases in LC employing CD bonded-phase stationary phases, retention is thought to be predominantly due to inclusion complex formation. The stability of the CD adducts varies markedly with the size of the CD cavity and/or with the structure of the substrate. Hydrogen bonding (4), van der Waals interactions (5, 6), hydrophobic interactions (6, 7), release of high-energy water (5), and com-

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Fig. 1. Biphenyl structure. The numbers indicate the positions of substitutents. α indicates the relative angle between the phenyl rings.
biphenyl as a result of its three-dimensional structure, CD-bonded phases were demonstrated to be particularly adept in resolving structural isomers (8) such as the ortho-, meta-, and para- forms of many monoaromatic compounds. We have previously reviewed the LC retention behaviour and separation of monoaromatic chlorophenols (1, 2). A few researchers have investigated the interactions of diaromatic compounds with CD-bonded stationary phases; Fujimura et al. (9) and Kawaguchi et al. (10) have extensively reported on the retention behaviour and separation of naphthalene and substituted naphthalenes on diaminob-CD and ethylenediamine-β-CD bonded-phase columns, respectively. Armstrong et al. (11) have described the separation of 80 compounds, including a number of diaromatic analogues, from their isomers using hydrolytically stable β-CD bonded-phase columns obtained from Advanced Separation Technologies, Inc., Whippany, NJ (12). Issaq et al. (13), using the same type of column, have examined the effect of mobile-phase composition, temperature, and pH on the retention of a selected group of compounds including naphthalene and biphenyl. Finally, Weaver and van Lier (14) have reported the separation of 2-, 3- and 4-hydroxybiphenyls on a β-CD bonded-phase column.

All studies discussed to this point dealt with the LC retention behaviour and/or separation, on a CD bonded-phase column, of structural isomers of relatively small diaromatic molecules with one or two substituents. Here we extend these investigations to structural isomers with more than one or two substituents on the benzene rings. A class of compounds that we found particularly suited for the study of diaromatic phenols and chlorophenols is the chlorinated and non-chlorinated biphenylols and biphenyldiols (PCB metabolic analogues). Sixteen commercially available members of this family of compounds were chosen for our studies (see Table 1). These compounds were chosen because they present a number of distinct features: (1) Biphenyl is non-planar in solution (15, 16) and the angle between the two benzene rings is affected by the nature and the position of the substituents (17) (see Fig. 1). Wide stereochemical diversity can exist in biphenyl as a result of its three-dimensional structure, especially when seen in combination with the diversity arising from the degree of substitution and spatial geometry of the substituents on either or both of the benzene rings. (2) The largest possible diameter of a chloro-substituted biphenylol is about 7.1 Å which is very close to the diameter of the β-CD cavity (7.8 Å). One would, therefore, expect a close fit for many of these compounds into the cavity. The length of unsubstituted biphenyl is 9.1 Å as compared to 7.8 Å for the length of the cavity. This would allow for maximum interaction with the cavity and possibly hydrogen bonding with the hydroxyls in the periphery of the cavity. (3) The pK_a's of the various biphenyl isomers vary depending on the position of the substituents and the number of substituents (see Table 1). Thus by varying the pH of the mobile phase one can examine both the chromatographic behaviour of both the neutral and anionic forms of these compounds. (4) There exists a large variation in the solubility of the various biphenylols in common reversed phase mobile phases. This difference is quite substantial between biphenylols and biphenyldiols.

This paper investigates the LC retention behaviour of chlorinated and non-chlorinated biphenylols and biphenyldiols on a β-CD column with respect to mobile-phase composition, pH, temperature and ionic strength. This behaviour is compared with the results previously obtained with the structurally simpler monoaromatic chlorophenols (1). Based on the findings of this study conditions were chosen for the isocratic and gradient-elution separation of these compounds.

Experimental

The LC system consisted of a Waters Model 590 pump, a Rhodyne Model 7125 injector with a 10-μL loop, and a Model Spectroflow 773 variable wavelength UV-vis detector equipped with a 8-μL cell. For the gradient-elution separations an additional Waters Model M-6000 pump was used along with a Waters Model 660 solvent programmer. The chromatograms were obtained by using a Microom Model SE 120 strip-chart recorder. The column temperature was controlled through a Heto Model 623 water bath.

A Cyclobond I, 250 x 4.6 mm column was obtained from Advanced Separation Technologies (Whippany, NJ). The Cyclobond I column has β-CD chemically bonded to a spherical silica gel support through a five-atom, non-nitrogen-containing spacer arm. The preparation and characterization of this column was fully described elsewhere (12). When not in use, the column was stored in 100% methanol. The column performance appeared to be unchanged for a period of about 3 months.

The effect of pH on the retention time of biphenylols was investigated by changing the pH of the aqueous part of the mobile phase from 4 to 6 using triethylamine-acetate buffers (0.01M). The effect of ionic strength on retention time was examined by varying the concentration of triethylamine buffer (pH = 4.0) in the aqueous part of the mobile phase from 0.01 to 0.1 M. The ionic strength study was performed at pH = 4.0 since at this pH all biphenylols are likely to be in their neutral form. The pK_a of all compounds, was determined from plots of retention time vs. pH on a PRP-1 premade analytical column obtained from Hamilton (18), in 60:40 (v/v) methanol:water.

The mobile phase was degassed by bubbling helium into it before use. The flow rate was 1.0 mL/min and the column back-pressure at this flow rate ranged from 1600 to 2200 psi. All compounds were dissolved in methanol to give concentrations of 0.02–0.1 mM. Typically 2 μL of these standard reference compounds were injected. The retention times and elution order were established from consecutive injections of the individual standards. The precision associated with the measured retention times for the various standards ranged from 5–10%. The wavelength of detection was 290 nm. Since almost all ions and molecules, including the typical volume indicators, such as Cl^-, Br^-, I^-, etc., partially bind to β-CD, they cannot be directly employed to determine column void volumes or times. For this reason, a special procedure by Hinze et al. (19) was used to estimate the void volume of the column which was found to be 3.1 mL at a flow rate of 1 mL/min.

HPLC grade methanol was obtained from Fisher Scientific Co., triethylamine from Aldrich and acetic acid from J. J. Baker Chemical Co. Water was deionized by passing distilled water through a Barnstead Water purification system. Biphenylol, biphenylidol, chlorobiphenylol, and chlorobiphenyldiol standards were purchased from Chem Service.

Discussion

Effect of mobile-phase composition

The effect of mobile-phase composition on the capacity factor of biphenylols, chlorobiphenylols, and biphenyldiols was investigated by changing the methanol–water ratio in the mobile phase from 40:60 to 100:0 (v/v). In this set of experiments the mobile phase contained no buffer.

It was previously shown that a linear relationship exists between the log of the capacity factor, k, and the volume fraction of methanol in methanol/water mixtures used as mobile phases in reversed-phase chromatography (20–22). The shapes of plots such as these are assumed to be due to structural variations in the solvent system as the concentration of the organic modifier is changed. The fact that in the case of metha-
Fig. 2. Effect of mobile-phase composition on the logarithm of the capacity factor for biphenylols. Conditions: column, Cyclobond I (β-cyclodextrin bonded-phase); no buffer was used in the mobile phase; flow rate 1 mL/min; \( t_0 = 3.1 \) min; ambient temperature.

By plotting \( \log k \) vs. methanol content in the mobile phase for the various biphenylols, chlorobiphenylols, and biphenyldiols we found that the curves obtained are mostly linear (see Figs. 2–4) as would be expected if a reversed mechanism of retention was in operation. This is not the case, however, for 3,3',5,5'-tetrachloro-4,4'-biphenyldiol, and 2',3',4',5' tetrachloro-4-biphenylol (see Fig. 3) which present local maxima at around 50% methanol. These maxima may be taken as suggestive evidence that in CD-bonded-phase chromatography one or more mechanisms of retention, in addition to the reversed-phase mechanism encountered with hydrocarbonaceous bonded-phases (hydrophobic interactions), may be in operation (e.g. van der Waals interactions of the chlorine substituents with the backbone of the cavity). Similar behaviour was observed by Chang and Wu (23) with nitrophenols, on a γ-CD column, with 2-propanol as the organic modifier and Paleologou et al. (1) with highly chlorinated monoaromatic chlorophenols, on a β-cyclodextrin column, with methanol as the organic modifier.

It should also be noted that for all biphenylols and particularly the more highly chlorinated ones, the shape of the peaks was affected by the mobile-phase composition; peaks became sharper (smaller width) as well as more symmetrical as the methanol content increased.

Effect of pH

The effect of pH on the retention time of biphenylols, chlorobiphenylols, and biphenyldiols was investigated by changing the pH of the aqueous part of the mobile phase (50/50: methanol/water) from 4 to 6 using triethylamine buffers (0.01 M). From plots of retention time vs. pH (e.g. see Fig. 5) it appears that for all compounds, except one, there is no change in retention time with pH in the range examined. This situation can be rationalized in terms of the \( pK_a \)'s of these compounds (see Table 1). It should be pointed here that one can expect marked
effects on the retention as long as the pH of the mobile phase is within one unit of the $pK_a$ of a given compound.

All compounds examined, except one, have $pK_a$ values which exceed the maximum of the pH range examined by more than one pH unit and therefore are expected to remain in their neutral form throughout this range. In the case of 3,3',5,5'-tetrachloro-4,4'-biphenyldiol, however (see Fig. 5), an increase in retention time is observed with increasing pH. This change can also be rationalized on the basis of the $pK_a$ of this compound (see Table 1) which is quite a bit lower than the rest of the chlorophenols ($pK_a = 6.0$). It should be remembered at this point that binding to the CD lowers the $pK_a$'s of chlorophenols (24) and therefore brings them closer to the pH range examined. Furthermore, since the mobile phase contains methanol, the apparent pH of the mobile phase is actually higher than that of the aqueous phase (25); these two effects are likely to position the compound closer to the pH range examined.

At pH = 4.0 all diaromatic phenols and chlorophenols are expected to be in their neutral form and therefore, valid comparisons can be made between these and the monoaromatic chlorophenols (1), under the same conditions, regarding the extent of their participation in the inclusion process. Table 1 presents the retention times of all diaromatic phenols under these conditions.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>$pK_a$</th>
<th>$t_R$ (min)</th>
<th>$t_K$ (min)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3,4-Biphenyldiol</td>
<td>9.3</td>
<td>5.6</td>
<td>4.0</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2,5-Biphenyldiol</td>
<td>11.0</td>
<td>5.8</td>
<td>4.4</td>
<td>-7.2</td>
</tr>
<tr>
<td>3</td>
<td>2,2'-Biphenyldiol</td>
<td>9.4</td>
<td>6.1</td>
<td>4.7</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>3,3'-Biphenyldiol</td>
<td>10.0</td>
<td>9.6</td>
<td>6.1</td>
<td>-9.8</td>
</tr>
<tr>
<td>5</td>
<td>2-Biphenyldiol</td>
<td>10.9</td>
<td>9.4</td>
<td>7.8</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>2',5'-Dichloro-2-biphenyldiol</td>
<td>11.0</td>
<td>9.3</td>
<td>8.8</td>
<td>-8.4</td>
</tr>
<tr>
<td>7</td>
<td>2',3,5'-Trichloro-2-biphenyld</td>
<td>9.0</td>
<td>10.7</td>
<td>11.3</td>
<td>-10.5</td>
</tr>
<tr>
<td>8</td>
<td>4,4'-Dichloro-3,3'-biphenyldi</td>
<td>8.3</td>
<td>10.8</td>
<td>11.3</td>
<td>-9.9</td>
</tr>
<tr>
<td>9</td>
<td>3',3',4',5,5'-Pentachloro-2-biphenyldiol</td>
<td>9.6</td>
<td>12.1</td>
<td>12.7</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>4,4'-Biphenyldiol</td>
<td>10.0</td>
<td>13.0</td>
<td>12.7</td>
<td>-14.0</td>
</tr>
<tr>
<td>11</td>
<td>3-Biphenyldiol</td>
<td>10.6</td>
<td>16.3</td>
<td>14.1</td>
<td>-9.5</td>
</tr>
<tr>
<td>12</td>
<td>4-Biphenyldiol</td>
<td>10.2</td>
<td>20.1</td>
<td>16.7</td>
<td>-8.9</td>
</tr>
<tr>
<td>13</td>
<td>2',3',4',5'-Tetrachloro-3-biphenyld</td>
<td>—</td>
<td>—</td>
<td>18.4</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>3-Chloro-4-biphenylol</td>
<td>8.6</td>
<td>22.7</td>
<td>19.2</td>
<td>-9.7</td>
</tr>
<tr>
<td>15</td>
<td>2',3',4',5'-Tetrachloro-4-biphenyldiol</td>
<td>10.1</td>
<td>24.1</td>
<td>25.9</td>
<td>-8.5</td>
</tr>
<tr>
<td>16</td>
<td>3,3',5,5'-Tetrachloro-4,4'-biphenyldiol</td>
<td>6.0</td>
<td>18.9</td>
<td>34.7</td>
<td>-13.6</td>
</tr>
</tbody>
</table>

*In 60:40 methanol/water.
*Retention time at pH = 4.0; $t_0 = 3.1$ mL.
*Retention time at gradient elution conditions; $t_0 = 3.1$ mL.
*In 50:50 methanol/water.

β-CD cavity sees a diameter (projected width) of 4.3 Å and therefore this molecule can very easily enter this cavity from end to end. The 3-substituted analogs, on the other hand, can penetrate the cavity to a limited extent because of the phenyl substituent at the 3-position; in this case the CD cavity sees a diameter of 7.9 Å which is slightly larger than the diameter of the opening of the CD cavity itself. The 2-substituted analogs are likely to enter the cavity and interact with it. Since, in fact, one ring is at a slight angle with respect to the other (15, 16), it is likely that the two rings interact differently with the cavity and probably in a complementary fashion. The retention time for 3-biphenyl is also significantly higher than that of 3-chlorophenol (16.3 vs. 6.2 min). If this molecule were to be entering the cavity with the —OH group first, at all times, then one would expect a retention time similar to 3-chlorophenol since only one of the two benzene rings would have entered the CD cavity and this only partially. It, therefore, appears that the cavity can also be entered by way of the unsubstituted benzene ring. The high retention time of 2-biphenyl as compared to 2-chlorophenol (9.4 vs. to 5.8 min) can be rationalized using a similar argument.
In the case of chloro-substituted biphenylols the following observations can be made regarding their retention behaviour. For 3-chloro-4-biphenylol, for example, the retention time is very similar to 4-biphenylol (22.7 vs. 20.1 min); this may be ascribed to the linear geometry of both these molecules which can allow full penetration into the cavity as illustrated above. The retention time is slightly higher in the former case because of the closer fit of one of the two benzene rings into the CD cavity as a result of the chlorine substituent; the cavity in the latter case sees a diameter of 5.64 \( \AA \) as compared to 4.26 \( \AA \) in the former case. Chlorine, as well as other substituents, are also known to affect the angle between the two benzene rings in biphenyl-type compounds (17), thereby leading to a three-dimensional interaction with the cavity. For 2',3',4',5'-tetrachloro-4-biphenylol the retention time is even higher (24.1 min) probably due to the increased interactions between the second benzene ring and the cavity because of its numerous chlorine substituents; the cavity sees a diameter of 7.03 \( \AA \) in this case, suggesting an almost perfect fit in the cyclodextrin cavity. A similar argument can be made to justify the greater retentions times of 2',3',4',5'-tetrachloro-3-biphenylol (d = 7.03 \( \AA \)) for that part of the molecule that can enter the CD cavity, \( t_R = 20.5 \) min as compared to 3 biphenylol (d = 4.26 \( \AA \), \( t_R = 16.3 \) min) and 2',3',5'-trichloro-2-biphenylol (d = 7.03 \( \AA \), \( t_R = 10.7 \) min) and 2',3',4,5,5'-pentachloro-2-biphenylol (d = 7.03 \( \AA \), \( t_R = 12.1 \) min) as compared to 2-biphenylol (d = 4.26 \( \AA \), \( t_R = 9.4 \) min). In the case of biphenyls hydroxylated in the 2-position, only one benzene ring is likely to enter the cavity at a time and therefore, these retention times are likely to reflect, to some extent, weighted averages of the retention times of the corresponding monoaromatic chlorophenols and chlorobenzenes.

**Effect of temperature**

The effect of temperature on the retention time of biphenylools, chlorobiphenylools, and biphenyliodols was investigated in the temperature range 20–60°C with a mobile phase that was 50:50 (v/v) methanol:water. In this set of experiments the mobile phase contained no buffer. In all cases a decline in retention time with increasing temperature is observed (e.g. see Fig. 6 for biphenylools). This is likely to follow the decrease in the binding constant to the \( \beta \)-CD with increasing temperature. The temperature effect appears to increase in the order biphenyliodols<chlorobiphenylools<chlorobiphenylols. Moreover, the decline in retention with temperature is quite regular suggesting the same mechanism of retention at different temperatures for any given compound. An exception to this trend is 3,3',5,5'-tetracloro-4,4'-biphenyliodol that presents very high retention times for temperatures below 40°C.

The effect of temperature on retention is largely determined by the enthalpy of eluite interaction with the stationary phase. The enthalpy can be evaluated from the slope of van’t Hoff plots, that is plots of log \( K \) versus the reciprocal of the absolute temperature (31, 32). When the equilibrium for eluite binding by the stationary phase can be written in terms of a single eluite species and a single bound species, then the enthalpy of this particular binding process is easily obtained. Figure 6 presents van’t Hoff plots for the three biphenylols. Since all three of these compounds as well as all other diaromatic chlorophenols, except one, present pK\(_A\)'s significantly above the \( \rho \)H of the mobile phase, a single eluite species and a single bound species is likely to exist. In addition, since these plots are quite linear, it is possible to determine the enthalpy of binding from the slope of the van’t Hoff plots. These values are given in Table 1. From the intercept of van’t Hoff plots it is theoretically possible to calculate the change in the corresponding entropy as well. This is difficult, however, because with bonded phases the phase ratio cannot be clearly defined (31). Given the relative bulkiness of biphenylools (4.26 \( \AA \) projected diameter, assuming that one ring enters the cavity) and especially substituted biphenylols (5.64 to 7.03 \( \AA \) depending on number and position of substituents) in comparison to the diameter of the \( \beta \)-CD cavity (7.8 \( \AA \) at the front and 6 \( \AA \) at the back) it is likely that the complexation is mostly governed by enthalpy and not entropy (33). This is because of the increased contribution or van der Waals interactions (\( \Delta H < 0, \ \Delta S < 0 \)) in the binding process. Among the binding forces proposed for inclusion phenomena only hydrophobic interactions are governed by entropy. These would be of significance only in the case of relatively small substrates. Evidence supporting this hypothesis is also provided by gas chromatography using \( \beta \)-CD as the stationary phase (34) and di- and tri-substituted benzenes as the substrates; the retention and selectivity are quite appreciable despite the complete absence of hydrophobic interactions.

Deviations from linearity do exist for some of these compounds (e.g. 2,5-biphenyliodol, 2',5'-dichloro-2-biphenyliodol, 4,4'-biphenyliodol, 4,4'-dichloro-3,3'-biphenyliodol and 3,3',5,5'-4,4'-biphenyliodol). Non-linear van’t Hoff plots in RPC can be expected whenever one of the following three conditions hold (31): (1) the eluite exists in two or more forms having different capacity factors; (2) the eluite is retained by two or more mechanisms due to the heterogeneity of the stationary phase surface containing more than one type of binding site; (3) eluite exists in more than one form and the surface is heterogeneous.

In the case of 2,5-biphenyliodol, for example, the non-linear van’t Hoff plot is likely to be due to condition (2) being in effect. At \( T = 40°C (10^3/T = 3.20) \) a break is observed in its van’t Hoff plot. This break is likely to correspond to the dissociation of the CD-2,5-biphenyliodol complex at this temperature. 2,5-Biphenyliodol is not likely to fully enter the cavity because it has a phenyl substituent in ortho - to one of the hydroxyl groups. Other biphenyliodols (e.g. 4,4'-) present breaks at a higher temperature (50°C) while the remaining ones present quite linear behaviour throughout the temperature range exam-
range between 0-0.05 M followed by a plateau in the 0.05-0.1 M range. Non-linear van’t Hoff plots on a main axis. The effect of ionic strength on biphenylols and aromatic chlorophenols. Conditions: column, Cyclobond I (β-cyclodextrin bonded-phase); mobile phase, 50:50 methanol:water (buffered to pH = 4.0 with triethylamine acetate); flow rate, 1 mL/min; $t_0 = 3.1$ mL; ambient temperature.

In the case of biphenylols is similar to that observed, in the case of biphenyldiols, the van’t Hoff plot is also non-linear. The curvature in this plot is likely to be due to condition (3) being in effect since the $pK_a$ of this compound is close to the pH of the mobile phase; this means that the eluite species is likely to exist in two forms (non-ionized and ionized). Since in the case of this compound there was more than one kind of eluite species, the van’t Hoff plot yielded the overall retention enthalpy which is a weighted average of the enthalpies for the binding of the species. Similar behaviour was previously observed in the case of monoaromatic tri-, tetra-, and pentachlorophenols with $pK_a$'s close to the pH of the mobile phase (1). No enthalpies of binding were calculated for those compounds whose van’t Hoff plots did not present any appreciable degree of linearity. Selectivity is affected very slightly, if at all, by changing the temperature. In this regard β-CD columns appear to behave like other reversed-phase columns (35).

**Effect of ionic strength**

The effect of ionic strength on the retention time of biphenylols and chlorobiphenylols was investigated in the concentration range of 0.01 to 0.1 M triethylamine-acetate (for aqueous part of mobile phase pH = 4.0) in a 50/50 methanol:water mobile phase. In the case of biphenylidols (see Fig. 7) an initial decline in retention is observed in the electrolyte concentration range between 0–0.05 M followed by a plateau in the 0.05–0.1 M range. The effect of ionic strength on biphenylols and chlorobiphenylols is similar to that observed; in the case biphenylidols, it is, however, more pronounced. An exception to the above behaviour is presented by 3,4-biphenyldiol whose retention increases in the 0.01–0.05 M ionic strength range while stabilizing in the 0.05–0.1M range. This phenomenon is likely to be due to the disruption of intramolecular hydrogen bonding in this compound by increasing ionic strength and the resulting increased interaction of its hydroxyls with the secondary hydroxyls at the periphery of the cavity; as a result of their electrical charge, both the triethylammonium cation and the acetate anion can be expected to be involved in the formation of more powerful hydrogen bonds with a given aromatic hydroxyl as compared to such a bond with an adjacent neutral hydroxyl (36).

Resolution is also affected by ionic strength. The peak shapes of chlorophenols and especially the most chlorinated ones, become sharper (shorter width) and more symmetrical with increasing buffer concentration. The selectivity of the separation of chlorophenols is affected very slightly, if at all, by the ionic strength as evidenced by the absence of any changes in the elution order of chlorophenols with increasing ionic strength. Since the presence of an electrolyte in the mobile phase does not affect the selectivity, it is possible to couple the β-CD bonded-phase column with an electrochemical detector (2).

Horvath et al. (37) showed that in RPC employing hydrocarbonaceous bonded phases, increasing ionic strength augments the capacity factors of neutral solutes. This effect has been ascribed to the increasing surface tension of the eluent and the concomitant increase in the energy required for cavity formation (38). As seen in Fig. 7, however, exactly the opposite behaviour is observed in the case of a CD-bonded silica stationary phase. This type of behaviour was previously encountered with monoaromatic chlorophenols as well and was rationalized in terms of the triethylamine cations including into the cavity thus competing with the substrate molecules (1).

**Separation**

After studying the liquid chromatographic retention behaviour of diaromatic chlorophenols with respect to mobile-phase composition, pH, temperature and ionic strength conditions were chosen for the isocratic separation of these compounds. Even though each category of chlorobiphenylols (2-, 3-, 4-) could be separated into its member components with reasonable resolution and selectivity, significant overlaps existed between the retention time ranges of the various categories which prevented the isocratic separation of all chlorophenols on the same chromatogram. A gradient-elution separation was, therefore, attempted.

Table 1 and Fig. 8 present the retention times and the gradient-elution separation, respectively, of 14 out of the 16 diaromatic chlorophenols within 35 minutes of injection of the mixture.
This separation was achieved with a linear mobile phase gradient of 1%/min increase in methanol (solvent B) beginning with a 27:73 methanol/water (solvent A) mobile phase. The temperature was 50°C, the buffer concentration 0.01 M triethylammonium acetate (TEAA) and the pH = 4.0. The design of this gradient was based on our previous experience with the gradient of chlorinated and non-chlorinated biphenylols and biphenyldiols. We have thus established in this paper that the column (2). We have thus established in this paper that the well-known selectivity of CD columns can be extended to substituted and non-substituted diaromatic phenols such as the chlorinated and non-chlorinated biphenylols and biphenyldiols.

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