FACILE SEPARATION OF ENANTIOMERS, GEOMETRICAL ISOMERS, AND ROUTINE COMPOUNDS ON STABLE CYCLODEXTRIN LC BONDED PHASES

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ABSTRACT. The effectiveness of employing stationary phases composed of chemically bonded cyclodextrin molecules in the high performance liquid chromatographic separation of a variety of different types of compounds is summarized. Over one hundred compounds, including optical, geometrical, and structural isomers, diastereomers, and epimers were successfully separated from each other via use of beta- or gamma-cyclodextrin bonded phases and aqueous methanolic mobile phases. The mechanism of separation is based upon inclusion complex formation between the compounds being separated and the cyclodextrin molecules bonded to the stationary phase. The effects of temperature, mobile phase composition and flow rate upon the chromatographic selectivity and resolution are described. The results indicate that the cyclodextrin columns may be more versatile, flexible, and effective compared to the conventional normal or reversed phase columns.

1. INTRODUCTION

Alpha-(α), beta-(β), and gamma-(γ) cyclodextrins are cyclic oligosaccharides consisting of six, seven, and eight D(+)-glucopyranose units, respectively, which have the shape of a hollow truncated cone, the interior of which forms a relatively hydrophobic cavity [1]. The unique ability of cyclodextrins to form inclusion complexes with a variety of substances has been advantageously utilized in many industrial, pharmaceutical, agricultural, and related applications [2]. The relative stabilities of such cyclodextrin inclusion complexes are governed by factors such as hydrogen bonding, hydrophobic interactions, solvation effects as well as the guest molecule's space filling ability [1-4]. Depending upon the size and geometry of a guest in relation to that of the cyclodextrin host, substantial differences in binding behavior can be observed for a series of structurally related solutes. This pro-

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vides the basis for their use in various chemical separation and purification schemes [5,6].

Cyclodextrins have previously been successfully employed in separation science. For instance, the partial separation and enrichment of optical and structural isomers as well as routine compounds based on selective precipitation with CDs have been reported [5,7-8]. Additionally, solutions of CDs have served as the mobile phase in a few thin-layer and high performance liquid chromatographic separations [5,9,10]. However, their most widespread application in chromatography has been as part of the stationary phase [5,6]. Various polymeric CD materials, CD gels or resins, as well as CD coated columns have been utilized as the stationary phases in the separation of many important classes of compounds [5,6,11-13]. Unfortunately, the use of these CD phases has been largely restricted to column or gas chromatography due to their low efficiency and/or poor mechanical strength [14-16].

More recently, several reports have appeared which describe the preparation of HPLC columns which contain CD chemically bonded to silica gel [15-18]. Of these, there are presently two types. The first consists of CD bonded to the silica via amide or amine bonds [15,16] while the second contains no nitrogen linkages [17,18]. This review article summarizes our chromatographic work to date with the latter type of CD bonded phases. In particular, we demonstrate the successful HPLC separation of enantiomers, epimers, cis-trans and other structural isomers as well as important classes of routine compounds by use of a β- or γ-CD bonded phase. The obtained chromatographic separations are compared to those obtained on the more conventional normal or reversed phase packings. Additionally, the effect of changes of the pertinent chromatographic variables (such as flow rate, temperature, and solvent composition of the mobile phase) upon the separations are described. Lastly, a brief prospectus on the future of CD bonded phases in HPLC is presented.

2. EXPERIMENTAL

All HPLC separations were performed using either a Shimadzu Model LC-4A liquid chromatograph with a variable wavelength detector containing a 13 μl flow cell (referred to as System I) or a Waters unit consisting of two Model 510 pumps, Model 680 automated gradient controller, and Model U6K injector equipped with a variable wavelength detector containing a 14 μl flow cell (referred to as System II). The β-CD columns (4.6 x 100 or 250 mm) and γ-CD column (4.6 x 100 mm) were obtained from Advanced Separation Technologies, Inc. (Whippany, NJ). These columns have β- or γ-CD molecules chemically bonded via a non-nitrogen or sulfur containing spacer to 5 micron diameter spherical silica gel. A complete description of the preparation and characterization of these columns is given elsewhere [17,18]. The performance of these columns appeared to be unchanged for a period of at least four months and they can be easily regenerated by merely flushing with ethanol or acetonitrile [22,23]. HPLC grade solvents were employed as the mobile phase components. The β-CD employed in the preparation of some mobile phases

was used as obtained from Advanced Separation Technologies, Inc. All other chemicals and reagents used were of the best available reagent grade materials. The complete description of exact chromatographic and experimental procedures employed can be found in the original cited references [17-22].

3. RESULTS AND DISCUSSION

3.1. Chromatographic Separations on the Chemically Bonded CD Phases

The chromatographic retention and selectivity data for the separation of over sixty compounds from their isomers is summarized in Tables I and II. These include many positional isomers (Table I, # 1-5), compounds that are structural isomers by virtue of either a translocation of an aromatic ring (Table I, # 6,7) or double bond (Table I, # 8,9), two classes of diastereomeric compounds, namely geometrical cis-trans isomers (Table I, # 10,11) and epimers (Table I, # 12,14), and optical isomers (Table II). In most instances, these isomeric compounds could be baseline resolved on the β-CD stationary phase using either aqueous methanol, ethanol, acetonitrile, or tetrahydrofuran as the mobile phase.

TABLE I. Retention Data for the LC Separation of Structural Isomeric and Diastereomeric Compounds on Cyclodextrin Bonded Phases

Compound	k'[a]	Conditions[b]	Mobile Phase[c]	Ref.
1. o-Cyanophenol	0.8 [0.2]	A	25%AN	d
m-Cyanophenol	0.5 [0.1]		[40%AN]	
p-Cyanophenol	0.7 [0.2]			
2. o-Nitroaniline	5.9	B	40%MeOH	18,
m-Nitroaniline	5.4			21
p-Nitroaniline	14.0			
3. 7-Methylindole	0.8	A	50%MeOH	21,
5-Methylindole	1.6			d
3-Methylindole	1.1			
2-Methylindole	1.4			
1-Methylindole	1.7			
Indole	1.3			
4. o-Cresol	2.3	C	30%MeOH	21
m-Cresol	1.9			
p-Cresol	3.5			
5. Quinoline	1.7 [0.9] (11.3)			
Isoquinoline	2.2 [1.1] (10.7)	A, (D)	40%MeOH [50%MeOH] (e)	21, d

(continued, next page)

TABLE I. Continued.

Compound	k'[a]	Conditions[b]	Mobile Phase[c]	Ref.
6. Benzo(a)pyrene	7.9	E	50%MeOH	18,
Benzo(e)pyrene	6.2			21
7. Phenanthrene	2.6	E	55%MeOH	21
Anthracene	3.4			
8. Prostaglandin A_2	4.6	E	50%MeOH	21
Prostaglandin B_2	2.0			
9. Vitamin D_2	0.4	C	90%MeOH	21
Lumisterol	0.7			
Previtamin D	1.2			
10. trans-Chlomiphene	3.6	F	65%MeOH	21
cis-Chlomiphene	5.4			
11. cis-Benzo(a)pyrene-7,8-diol	18 [19]	F, [G]	35%MeOH [30%MeOH]	20, 21
trans-Benzo(a)pyrene-7,8-diol	20 [24]			
12. 5β-Androstan-3β-ol-17-one	2.0	E	75%MeOH	21
5α-Androstan-3β-ol-17-one	10.1			
5α-Androstan-3α-ol-17-one	1.8			
5β-Androstan-3α-ol-17-one	6.4			
13. d-Aldosterone	5.2	E	40%MeOH	21
17-Isoaldosterone	4.9			
14. Estriol	3.8	E	60%MeOH	20,
16-Epiesteriol	11.5			21
17-Epiesteriol	5.8			
16,17-Espiesteriol	2.8			

[a]Capacity factor calculated using methanol as the retention marker.

[b]Conditions: A = chromatographic system II, 25-cm β-CD column, flow rate = 1.0 ml/min. B = chromatographic system I, 10-cm β-CD column, flow rate = 1.0 ml/min. C = chromatographic system I, 25-cm β-CD column, flow rate = 1.0 ml/min. D = chromatographic system II, 10-cm β-CD column, flow rate = 1.0 ml/min. E = chromatographic system I, 10-cm β-CD column, flow rate = 1.5 ml/min. F = chromatographic system I, 25-cm β-CD column, flow rate = 1.5 ml/min. G = chromatographic system I, 10-cm γ-CD column, flow rate = 1.5 ml/min.

[c]Numbers refer to the percent volume of organic solvent in water. AN = acetonitrile and MeOH = methanol.

[d]This work. [e]Mobile phase consisted of aqueous 8.0×10^{-3} M β-CD containing 4.5% added methanol.

A typical chromatogram illustrating the type of separation achieved is shown in Figure 1 (separation of the cis and trans isomers of 3-hexen-1-ol and α- and β-isomers of naphthoflavone). It should be pointed out that many of the isomeric compounds were separated using a 10-cm column, so that even better resolution could have been achieved by using a longer 25-cm column and/or altering the mobile phase composition (Table II, # 2,3) or flow rate (Table II, # 9). Some compounds, such as the cis-trans isomers of benzo(a)pyrene-7,8-diol, which could not be baseline resolved on the β-CD column, could be completely resolved by merely switching to a γ-CD column (Table I, # 11).

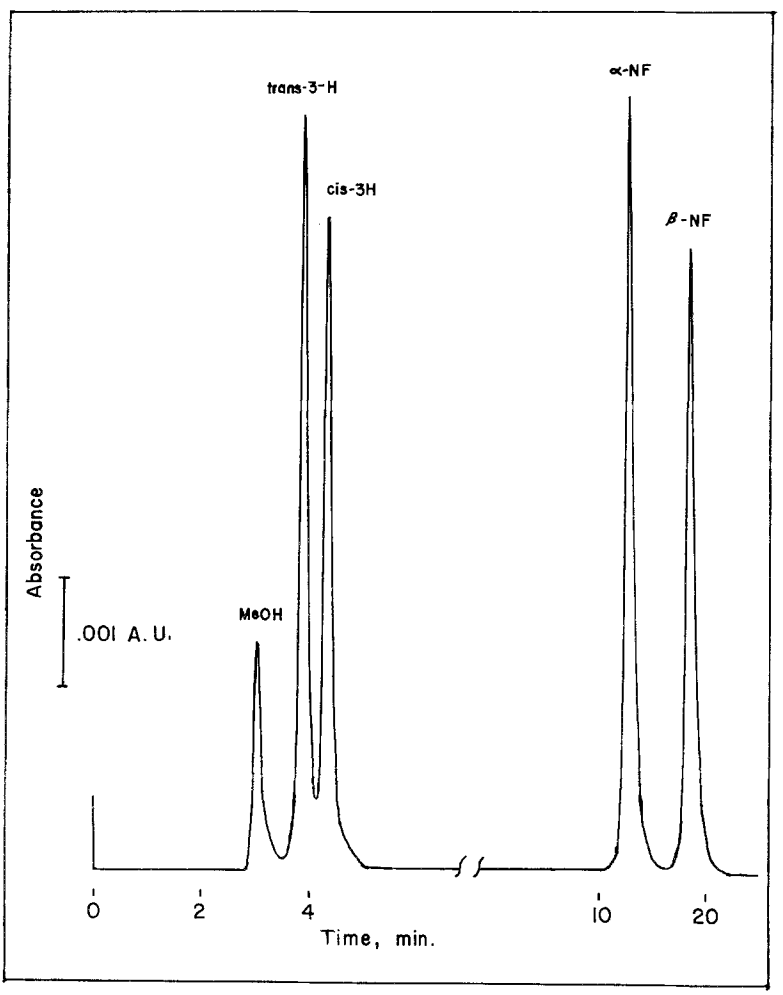

Figure 1. Chromatogram showing the separation of the cis and trans isomers of 3-hexen-1-ol (cis/trans 3-H) and α- and β-naphthoflavone (NF) on a 25-cm β-CD column using 50% aqueous methanol as the mobile phase. [Conditions: flow rate = 1.0 ml/min., 24° C, and chromatographic system II. The wavelength for detection of the 3-hexen-1-ols was 220 nm while that for the naphthoflavones was 273 nm.]

The results to date indicate that the cyclodextrin bonded phases are particularly adept at separating various types of structural isomeric compounds [5,13,15-18,20,21]. In addition to those substances listed in Table I, successful separations have also been achieved for the ortho, meta, and para isomers of aminobenzoic acid, bromobenzoic acid, nitrophenol, biphenyl, and xylene [18,21]; the α-/β-epimers of the steroids 20-hydroxy-4-pregnen-3-one, 5-androstan-3,17-dione, 17-estradiol, and 11-hydroxyprogesterone [21]; the α- and β-iosmers of naphthol [18], ethylphenethyl alcohol [21], and naphthoic acid [23]; the cis-trans isomers of stilbene [21]; and the following structural isomeric pairs: 7-epitestosterone/testosterone [21] and syn- and anti-azobenzene [21]. Preliminary results indicate that the isomers of o-, m-, and p-cis-nitrocinnamic acid, trans-nitrocinnamic acid, and ethyltoluene as well as the 1,2,3-, 1,2,4-, and 1,3,5-trimethylbenzenes appear to be easily resolved on the CD bonded phases [23]. These particular isomeric compounds (as well as others such as o-, m-, and p-xylene or cresol and benzo(a)pyrene and benzo(e)pyrene, Table I) are known to be extremely difficult to separate by LC (or GC) even when using gradient elution and optimized conditions [13,18,20,21]. Whereas all four epimeric estriols had not been previously resolved on conventional phases, they were easily baseline separated on a β-CD column (Table I, # 14) [21]. Consequently, the use of CD bonded phase columns in HPLC seems to be superior to the conventional normal or reversed phase packings that have been employed for the attempted separation of many of these types of isomeric compounds [18,21].

Perhaps the most exciting feature of the CD molecule is that each of its glucose units contain five chiral atoms, thus allowing these CD columns to function as chiral stationary phases (CSP) in the HPLC separation of enantiomers [5,18,19,22]. Table II summarizes some of the chromatographic retention, selectivity, and resolution data for the enantiomeric separations that have been obtained for some dansylsulfonamide, β-naphthamide, or β-naphthyl ester derivatives of amino acids, substituted dioxolanes and barbiturates using a β-CD column [19]. As can be seen, the baseline separation ($R_S > 1.5$) of many of these enantiomers was possible. In many cases, it was possible to detect as little as 0.20% of one enantiomer in the presence of 99.80% of the other [18]. Also, other enantiomers of dioxolanes, dansylamino acids [18,19] as well as the optically active alkylsubstituted naphthylsulfinates [23] have been partially resolved on CD columns using aqueous alcohol or acetonitrile mobile phases. More recently, the partial resolution of some enantiomeric aromatic carboxylic acids was reported using the Type I (nitrogen linkage in spacer group) β-CD column [24]. Taken together, these results clearly indicate the potential usefulness of these CD columns in HPLC optical resolutions. They appear to offer a viable alternative to the use of the more expensive, less versatile CSP now employed for the separation of many of the types of enantiomers listed in Table II [22].

The effectiveness of the CD columns in LC is not merely restricted to the separation of structural isomers, enantiomers, or diastereomers. For example, the complete baseline separation of a series of barbiturates (including barbital, butabarbital, sodium pentabarbital, pheno-

TABLE II. Retention, Selectivity, and Resolution Parameters for the LC Separation of Enantiomers upon the Beta-Cyclodextrin Column

Compounds	k'_1[a]	α	R_s	Mobile Phase[a]
1. Dansylthreonine	1.7	1.2	2.0	50% MeOH[b]
	2.4	1.2	1.0	30% AN[c]
2. Dansylphenylalanine	3.1	1.2	1.1	55% MeOH[b]
	6.1	1.1	d	30% EtOH[c]
	1.4	1.2	0.7	40% AN[c]
3. Dansylleucine	3.0	1.4	2.4	50% MeOH[b]
	4.0	1.2	0.7	30% EtOH[c]
	2.9	1.2	0.9	30% AN[c]
4. Alanine β-naphthyl ester	1.0	1.8	2.6	50% MeOH[b]
5. Alanine β-naphthamide	5.1	1.2	2.0	50% MeOH[b]
6. Mephobarbital	6.6	1.2	1.6	20% MeOH[e]
7. Hexobarbital	9.4	1.1	1.5	15% MeOH[b]
8. α-Methoxy-α-trifluoromethylphenylacetic acid	7.5	1.3	0.6	50% MeOH[c]
9. DDDD[f]	0.6	1.2	0.8[c] 1.0[g] 1.1[h] 1.2[i]	50% MeOH

[a]The capacity factor given refers to the first eluting enantiomer. The numbers for the mobile phase refer to the percent of organic solvent in water (v/v). AN = acetonitrile, MeOH = methanol, and EtOH = ethanol.
[b]Data obtained on chromatographic system I using a 10-cm column and flow rate of 0.5 ml/min. [c]Data obtained on chromatographic system II using a 25-cm column and flow rate of 1.0 ml/min. [d]Not resolved.
[e]Data obtained on chromatographic system I using a 25-cm column and flow rate of 1.0 ml/min. [f]DDDD = Trans-α,α-(2,2-dimethyl-1,3-dioxolane-4,5-diyl)bis-(diphenylmethanol). [g]Flow rate = 0.6 ml/min.
[h]Flow rate = 0.4 ml/min. [i]Flow rate = 0.3 ml/min.

barbital, secobarbital, and amobarbital) on a β-CD column [18]; mycotoxins (such as T-2 tetraol, verrucurol, T-2 triol, HT-2 toxin, T-2 toxin, and diacetoxyscirpenol) on a β-CD column [20]; polycyclic aromatic hydrocarbons (including benzene, naphthalene, fluorene, phenanthene, biphenyl, fluoranthene, acenaphthene, benzo(a)pyrene, benzo(e)pyrene, 1,2,3,4-dibenzanthracene, and 1,2,5,6-dibenzanthracene) on both a β- and γ-CD column [20]; amino acids and simple dipeptides on a β-CD column [23]; as well as a series of heterocyclic aromatic compounds (Figure 2), vitamins, and quinones (Table III, # 1, 2) [20,23] on a β-CD column have been reported. As can be seen, a variety of conventional separations involving important classes of routine compounds can be efficiently performed on the CD columns. Hence, it appears that they may provide a useful alternative to the popular reversed phase packings

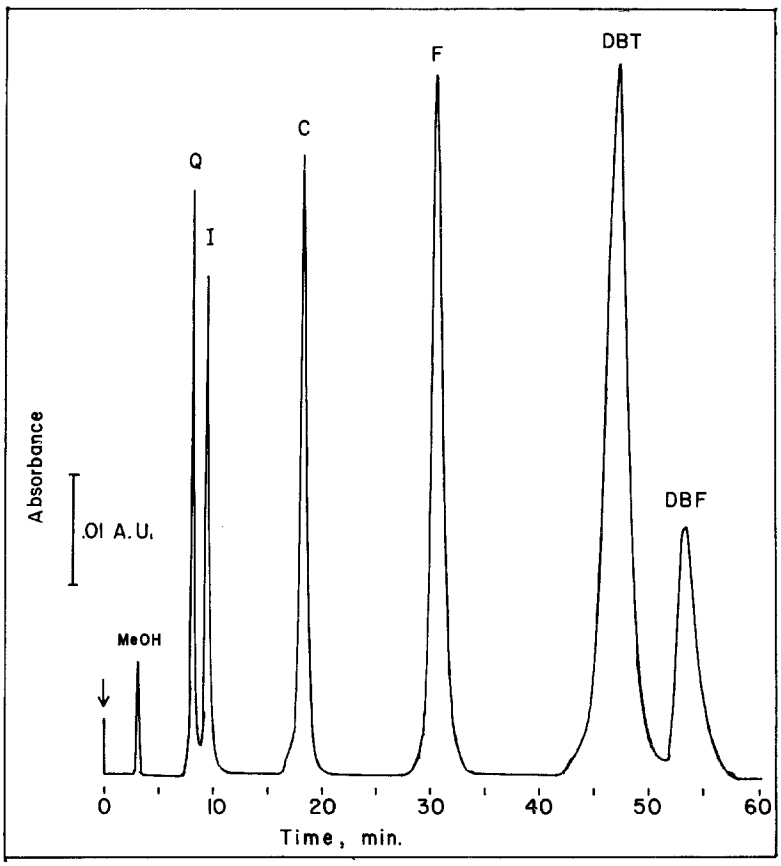

Figure 2. Chromatogram showing the near baseline separation of a series of heterocyclic aromatic compounds; quinoline (Q), isoquinoline (I), carbazole (C), fluorene (F), dibenzothiofuran (DBT), and dibenzofuran (DBF); on a 25-cm β-CD column using a 50% aqueous methanol mobile phase. [Conditions: chromatographic system II, 22° C, flow rate = 1.00 ml/min. The wavelength of detection for the quinolines was 223 nm while that for the other bridged heterocycles was 250 nm.]

and/or can be employed to compliment, if not replace, such conventional columns in the future for most routine separations [20].

Remarkably, preliminary data also suggest that cyclodextrin columns can be advantageously employed to separate different classes of anionic and cationic surfactants as well as inorganic ions [23]. For example, the data in Table III (entry # 3) indicate that the baseline separation of halide ions is achievable on the β-CD column. Therefore, it appears feasible that CD bonded phases can be used in lieu of the more expensive ion exchange columns currently required for the HPLC separation of such ionic species. The versatility of this particular

TABLE III. Retention, Selectivity, and Resolution Parameters for the LC Separation of Routine Substances on the β-CD Column[a]

	Compounds	k'	α	R_s	Mobile Phase[b]
1.	p-Benzoquinone	0.28 [0.25]			40% MeOH
			1.4 [1.3]	1.3 [0.8]	[45% MeOH]
	2,5-Dimethyl-benzoquinone	0.42 [0.33]			
			2.2 [2.0]	3.6 [2.2]	
	Duroquinone	0.94 [0.67]			
			1.0 ---	--- ---	
	1,2-Naphthaquinone	0.94 [0.72]			
			1.2 [1.2]	1.5 [1.0]	
	1,4-Naphthaquinone	1.16 [0.90]			
			4.4 [3.7]	8.9 [7.2]	
	Anthraquinone	5.09 [3.20]			
			--- [2.9]	--- [4.8]	
	Anthraquinone-1,5-disulfonic acid	--- [9.4]			
2.	Vitamin B_2	0.40 [0.80]			50% MeOH
			1.5 [3.2]	--- ---	[30% MeOH]
	Vitamin K_5	0.60 [2.6]			
3.	Chloride ion	0.6 [0.3]			75% MeOH
			4.2 [7.3]	--- ---	[50% MeOH]
	Bromide ion	2.5 [2.2]			
			1.4 [1.5]	--- ---	
	Iodide ion	3.5 [3.4]			

[a]All data obtained on chromatographic system I using a 25-cm column and [b]a flow rate of 1.00 ml/min. at 24° C.
[b]Refers to the volume percent of methanol to water in the mobile phase.

application needs to be demonstrated.

3.2. Dependence of Chromatographic Retention on Experimental Variables

Although the CD bonded phases are stable in a variety of different solvents including water and aqueous-organic mixtures (organic component = methanol, ethanol, tetrahydrofuran, or acetonitrile), the best separations were consistently obtained using methanol as the organic co-solvent (refer to Table II, # 1-3) [18,23]. This is presumbably due to the relative small degree of interaction between the CDs and the organic co-solvent methanol, compared with the other organic co-solvents [5, 19]. For all separations involving organic species, increases in the concentration of the organic component in the aqueous mobile phase result in decreases in the capacity factor and resolution. This general

behavior is shown by the data in Table III (entry # 1) and also in Figure 3, which shows a plot of k' (and R_S) vs. the percent methanol in the mobile phase for the separation of the enantiomers of DDDD. Data in Table III (entry # 3) also show that for ionic species, such as the halide ions, increases in the percent methanol in the mobile phase result in increased capacity factors, probably due to solvation requirements. This approach for altering capacity factors and resolution when using the CD columns is similar to that employed in reverse phase LC (e.g. controlling the concentration of an organic modifier). It should be emphasized however, that the primary mechanism of separation with CD columns (i.e. inclusion complex formation) if very different from that in reversed phase LC.

The CD columns are also effective when the mobile phase contains pure water (although the observed retention times are very large) or aqueous cyclodextrin (Table I, # 5), thus demonstrating their high de-

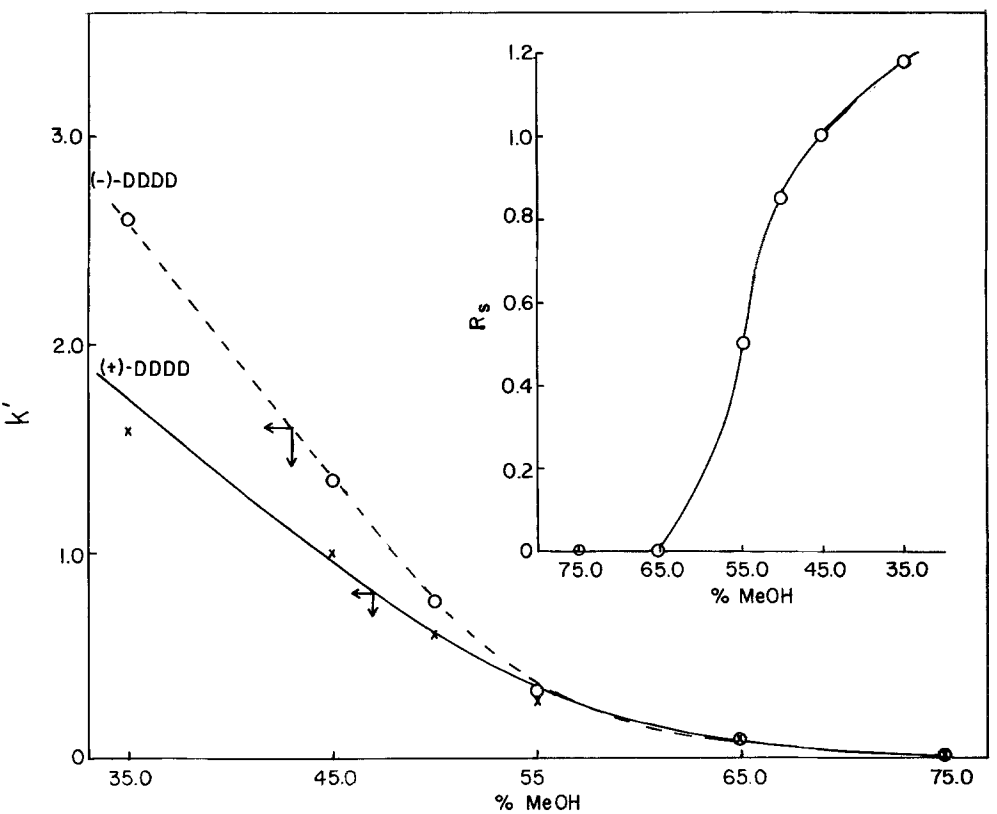

Figure 3. Graph showing the influence of percentage methanol in the aqueous mobile phase on the capacity factor, k', for the (+) and (-) enantiomers of DDDD on a 25-cm β-CD column. The inset to Figure 3 shows the influence of the percent methanol on the resolution of these enantiomers. [Conditions: chromatographic system II, 24.0° C, and flow rate of 1.00 ml/min.]

gree of versatility [23]. For aqueous CD mobile phases, the capacity factor decreases as the CD concentration increases [23]. These mobile phases provide the significant added advantages of allowing another means by which to control and manipulate chromatographic selectivity as well as allows for the possibility of utilizing room temperature liquid phosphorescence detection in HPLC [5,23,25].

The resolution of any pair of closely eluting species on the CD phases was found to be inversely proportional to the mobile phase flow rate. The greatest degree of improved resolution was generally observed when the flow rate was decreased from 1.50 ml/min to 0.40 ml/min. Further reductions in the flow rate caused only a small gain in the resolution (Table II, # 9) [19]. Hence, for optimum separation and resolution, a flow rate of 0.40 ml/min is recommended when using the CD columns described in this review. Although most separations shown herein were obtained under isocratic elution conditions at constant flow rates, appropriate use of solvent or flow gradients can in many instances speed up and/or enhance the separations [18,23].

The capacity factor and degree of resolution can be altered not only by the manipulation of the mobile phase but also by the type of CD column employed. Thus, α-, β-, and γ-CD exhibit different trends in their ability to form inclusion complexes with a particular series of solutes due to their different cavity dimensions [5]. Although most separations reported in this work were obtained using the β-CD column, comparisons can be made between certain separations obtained on both the β- and γ-CD columns. For example, the selectivity factor for benzo(a)pyrene (relative to acenaphthene) improves from 0.45 on a γ-CD column to 1.30 on a β-CD column [20]. Conversely, baseline resolution of the cis and trans isomers of benzo(a)pyrene-7,8-diol (Table I, # 11) was achieved with the γ-CD column whereas they were only partially resolved with the β-CD column [21]. CD molecules are easily obtained in three different sizes. One should thus be able to resolve a variety of different size solutes, ranging from small inorganic ions to molecules as large or larger than benzopyrene derivatives, by selecting the CD bonded phase which has the appropriate cavity dimensions for the HPLC separation at hand.

It is well known from CD solution studies that the strength of binding between a solute molecule and CD host increases as the temperature decreases [5]. Consequently, the capacity and selectivity factors increased as the temperature at which the HPLC separation was affected decreased [19,23]. However, the resolution factor did not correspondingly improve, due to diminished mass transfer at the lower temperature. For most separations, the use of the CD columns in HPLC at room temperature (20 - 25^{o} C) appears to be ideal. In some situations, however, increased column temperature can be beneficial since it results in improved efficiency (i.e. sharper peaks) and diminished analysis time [19,23,26].

4. CONCLUSIONS

The chemically bonded cyclodextrin stationary phases proved to provide

efficient, selective separations for a wide variety of isomeric components, routine compounds, and ions. Thus, the CD columns appear to be more widely applicable than either normal or reversed phase packings. Indeed, isomers and compounds that cannot be well resolved on these more conventional columns are often easily separated on the CD packings. The separation mechanism is primarily based on inclusion complex formation and is responsible for the unique but often predictable elution patterns observed [5,18,20]. Consequently, the use of such CD columns enhances the probability of using HPLC to predict or verify molecular structure [21]. The use of CD columns also appears to offer advantages in terms of experimental flexibility compared to popular columns now used for some special types of separation problems (i.e. enantiomeric resolution) [19,22]. The only apparent disadvantage of CD columns is their slightly diminished efficiency due to poorer mass transfer [18]. However, this somewhat reduced efficiency is more than offset by the spectacular enhancements in selectivity and resolution obtained by their use in HPLC. Also, much greater efficiency can be achieved by operating the CD columns at higher temperatures [23]. Due to the fact that different size-types of CD molecules as well as a variety of different spacer groups can be used in column design, the future use of cyclodextrin bonded phases holds great promise for the HPLC resolution of many other types of compounds.

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