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SAMPLE SOLVENT EFFECTS IN AN APPARENT CHIRAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION ON β-CYCLODEXTRIN

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SUMMARY

The effect of sample solvent on chromatographic peak shape using a β-cyclodextrin column has been investigated. Sample solvents ranging in polarity from hexane to mobile phase (water–methanol–0.5 *M* pH 4.5 borate buffer, 600:400:1) were used with assessments of column efficiency made by calculating theoretical plates as $N_{5\sigma}$, $N_{4\sigma}$ and N_{SYS}. Best agreement with observed peak shape resulted from use of $N_{5\sigma}$ especially in the narrow polarity span from 40 to 100% methanol. No chiral separation was obtained on the cyclodextrin column for the aminoalkylindole compounds investigated, although one possessed a naphthaldehyde substituent which should have promoted such separation.

INTRODUCTION

The use of chiral stationary phases (CSPs) in high-performance liquid chromatographic (HPLC) separations of enantiomers has grown rapidly with recent developments along several lines of column technology. These have been reviewed recently with indications of future applicability[1–5]. Commercially available, the Pirkle stationary phases have provided separation of amines, alcohols, amino alcohols, amino esters and amino amides. Another commercially available CSP is the bonded cyclodextrin column which has been applied to separate enantiomers of mandelic acid derivatives[6], aromatic carboxylic acids[7,8], naphthylamide, dansyl and benzoyl amino acids, barbiturates and dioxalanes[9,10]. β-Cyclodextrin (β-CD) bonded to a polyether polymer has also been used in separating isomers of indole alkaloids[11]. Derivatization of enantiomers is generally necessary prior to chiral separation on either Pirkle or β-CD columns to give dansyl, naphthyl, benzoyl, dinitrobenzoyl or similar functionalities.

The effect of sample solvent on chromatographic peak shape can be major or minor and is often perplexing to the practicing chromatographer. It can be the source of differences in results calculated by peak height *versus* peak area measurements by lab automation systems and should be accounted for in all quantitations requiring precision and accuracy. Use of mobile phase as sample solvent is often sufficient to

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overcome these potential problems while it may not necessarily be the optimal solvent as far as concentration–peak response is concerned. Occasionally because of sample solubility considerations it is not possible to use mobile phase as a sample solvent and a more or less polar solvent is substituted which can affect peak shape.

The effect of sample solvent on chiral separations using a β-CD column has not been reported although the source of normal-phase chromatographic peak broadening and splitting has been ascribed to sample solvent polarity differences with the mobile phase, sample solvent volume injected and flow-rate[12–14]. A dynamic adsorption–desorption model was used in one of these studies[13] to account for observed peak splitting based on competition between the sample solvent and mobile phase for adsorption sites.

Sample solvent polarity differences from the mobile phase have also been implicated in reversed-phase peak broadening and splitting[15,16]. While in an ion-pairing reversed-phase system, peak splitting was said to be due to competition between two different retention mechanisms, ion pairing and dynamic ion exchange[17], peak broadening for cyclosporine was shown in another study to result from various conformational isomers[18]. The effect of amine modifiers on peak symmetry in the reversed-phase retention of amine solutes was shown to result from characteristics associated with hydrogen bonding to silanol sites on the silica gel support[19].

In order to measure column efficiency as related to peak broadening and splitting effects the expression for number of theoretical plates calculating peak width at 4.4% peak height (5σ), eqn. 1 can be used[20,21].

$$N_{5\sigma} = 25\,(t_R/w_{4.4})^2 \tag{1}$$

Here t_R is the retention time and $w_{4.4}$ is the peak width at 4.4% of its height. Values calculated from this expression can be contrasted to the number of theoretical plates calculated from the usual eqn. 2 or its equivalent, eqn. 3.

$$N_{4\sigma} = 5.54\,(t_R/w_{0.5})^2 \tag{2}$$

$$N_{4\sigma} = 16\,(t_R/w)^2 \tag{3}$$

where $w_{0.5}$ is the peak width at one-half the maximum height and w is the peak width at its base.

Another measure of column efficiency is N_{SYS} proposed by Foley and Dorsey[22] which is calculated as

$$N_{SYS} = \frac{41.7\,(t_R/w_{0.1})^2}{b/a + 1.25} \tag{4}$$

where $w_{0.1}$ is the peak width at one-tenth of the maximum peak height, and a and b are distances measured at the same 10% peak height from the leading edge to the perpendicular dropped from the peak maximum and the distance from this perpendicular to the tailing edge of the peak respectively.

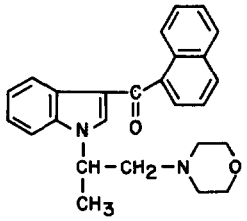
In addition peak asymmetry can be calculated using eqn. 5 (ref. 23), for peak symmetry, or eqn. 6 (ref. 24) for tailing factor

$$S = \frac{b}{a} \tag{5}$$

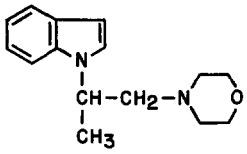
$$T = \frac{a + b}{2a} \tag{6}$$

where a and b have the same meanings as above except that in eqn. 6 they are measured at 5% maximum peak height.

The present study is an investigation of the sources of an observed peak splitting phenomenon on a β-CD bonded phase originally thought to be a chiral separation. Compounds included in this study are {1-[1-methyl-2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl}-(1-naphthalinyl)methanone, I, and its parent compound 1-[1-methyl-2-(4-morpholinyl)-ethyl]-1*H*-indole, II. The former should have been a good candidate for separation on a β-CD column according to published accounts although the indole group alone substituted on a chiral carbon could have given a separation of enantiomers on this column.



(I)



(II)

EXPERIMENTAL

Apparatus

A modular HPLC system was used consisting of a Waters 6000A pump, a Waters 440 UV absorbance detector operated at 254 nm, a Rheodyne 7125 injection valve with a 20-μl sample loop and a Fisher Recordall 5000 strip-chart recorder. The columns used were a β-CD (Cyclobond I), 25 cm × 4.6 mm I.D. (Astec, Whippany, NJ, U.S.A.), a Partisil PXS ODS-3, 25 cm × 4.6 mm I.D. (Whatman, Clifton, NJ, U.S.A.) and a Pirkle 1A ionic D-phenylglycine column, 25 cm × 4.6 mm I.D. (Regis Chemical, Morton Grove, IL, U.S.A.).

Chemicals and reagents

(+/−) I and (+/−) II were from Sterling-Winthrop Research Institute. The following chemicals were reagent grade: boric acid and copper(II) sulfate from Mallinckrodt, L-phenylalanine from Chemical Dynamics and D-camphorsulfonic acid from Eastman-Kodak. HPLC grade solvents from J. T. Baker were used including methanol, methylene chloride, acetonitrile, hexane, 2-propanol and chloroform while butyl chloride was HPLC grade from Burdick and Jackson. Water was Nanopure from a Sybron/Barnstead Nanopure II system.

Mobile phases

The mobile phase used with the β-CD and the ODS-3 columns consisted of water–methanol–0.5 *M* pH 4.5 borate buffer (600:400:1). Hexane–2-propanol, 95:5, 90:10 and 85:15 mobile phases were used with the Pirkle 1A column. Reversed-phase chiral mobile phases consisted of methylene chloride–acetonitrile–D-camphorsulfonic acid (60:40:0.005 *M* overall) to (20:80:0.05 *M* overall) and L-phenylalanine–Cu^{2+} (0.005 *M*:0.0025 *M* overall) and (0.010 *M*:0.005 *M* overall) in water. A flow-rate of 1.0 ml per min was used in all studies except in the reversed-phase ODS-3 study where 0.7 ml per min was used.

Sample preparation

Samples were prepared in mobile phase, hexane, butyl chloride, 2-propanol, chloroform, methanol, 90%, 80%, 70% and 60% (v/v) methanol in water at about 0.04 mg/ml each.

RESULTS AND DISCUSSION

The attempted chiral separation of (+/−) I began with use of a Pirkle 1A ionic chiral column with the racemate dissolved in hexane. Mobile phase variation from 95:5 to 85:15 (hexane–2-propanol) gave no separation of isomers for either the naphthyl ketone derivative I, or the parent II. Similarly use of the chiral mobile phase containing D-camphorsulfonic acid in methylene chloride–acetonitrile on a reversed-phase column was unsuccessful in separating racemic I or racemic II. This method had previously been used for a methylphenidate chiral resolution by diastereomeric ion-pair formation in the mobile phase[25]. Another reversed-phase chiral separation method involving charge transfer complex formation in the mobile phase containing copper(II) ions and phenylalanine did not give separation of enantiomers of II. This method has proven useful for resolution of optical isomers of a guanine derivative[26].

The apparent separation of enantiomers of I on the β-CD column using the water–methanol–borate buffer mobile phase is shown in Fig. 1A. This was obtained when the sample was dissolved in methanol as recommended by previous reports. The fact that a single peak resulted when the sample was prepared in mobile phase, as shown in Fig. 1B, and that chromatograms identical to Fig. 1A were obtained when methanolic solutions of the separate (+) and (−) isomers of I were run alone, revealed that the separation in Fig. 1A was an artifact. This could have resulted from a mixed retention mechanism involving both reversed-phase partitioning and chiral retention by the cyclodextrin bonded phase or from kinetic factors which were accentuated by sample solvent conditions.

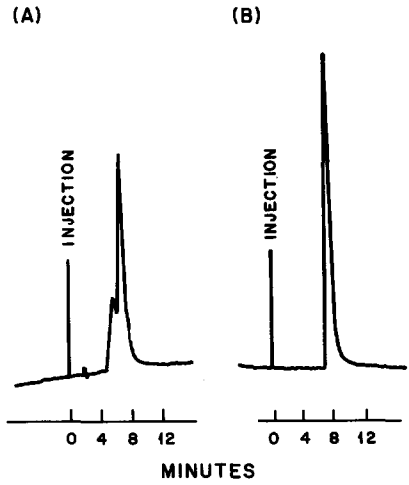


Fig. 1. Chromatograms of I at 0.04 mg/ml using a β-cyclodextrin column with the sample solvents (A) methanol and (B) mobile phase.

To indicate that the observed peak splitting did not result from a degree of reversed-phase partitioning inherent in the β-CD column, retention on an ODS-3 column with the same water–methanol–borate buffer mobile phase was studied. This possibility was investigated since separations on a β-CD column can be modified in much the same way as a reversed-phase column according to the manufacturer. Variation in methanol–water content of the mixed mobile phase can be used to give appropriate retention times for chromatographic peaks. Fig. 2A and B are chromatograms of solutions of (+/−) I in methanol and mobile phase respectively on the ODS-3 column where no splitting or shifts in retention time were seen. This indicated that the observed peak splitting on β-CD was due to a kinetic relationship between the sample in its solvent and the unique structure of the bonded phase which gives the enantio-separating power to the column.

The effect of sample solvent polarity was studied by dissolving the naphthyl ketone derivative, I, in solvents ranging from mobile phase to hexane. Results in terms of column efficiency calculated as $N_{5\sigma}$, $N_{4\sigma}$ and N_{SYS} are shown in Table I. While extremely low theoretical plate values were obtained from all three equations, it is clear that no simple relationship is followed between sample solvent polarity and column efficiency at least over this wide range of polarities. Best agreement between column efficiency and qualitatively observed peak shape was obtained by use of $N_{5\sigma}$ where the much improved peak shape using mobile phase rather than methanol as sample solvent corresponded to a near tripling of theoretical plates. Conversely, using either $N_{4\sigma}$ or N_{SYS}, a higher plate count was found for samples injected from methanol in contrast to direct observation of peak shapes.

This points out the artificially high plate count resulting from the assumption of Gaussian peak shape in eqn. 2 as well as the low order of correspondence between peak width measured at half-height and overall peak shape. This agrees with previous work by Vivilecchia *et al.*[20] on gel permeation peaks where $N_{5\sigma}$ was shown to be more closely related to peak shape than $N_{4\sigma}$. In the same way the use of eqn. 4 to

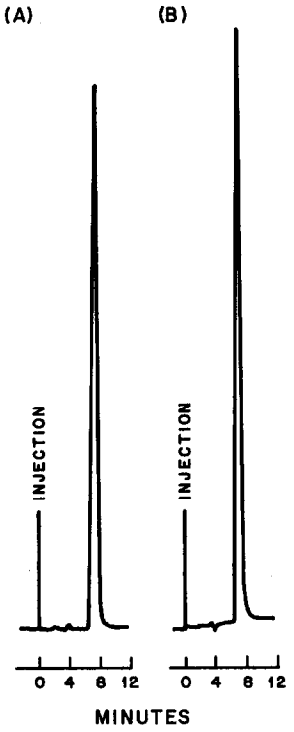


Fig. 2. Chromatograms of I at 0.04 mg/ml using an ODS-3 column with the sample solvents (A) methanol and (B) mobile phase.

calculate N_{SYS} is based on the assumption of exponentially modified gaussian (EMG) peak shape[22]. The values of N_{SYS} related well to results calculated for skewed peaks in that study and it gives a higher order of correspondence with the observed peak shape in the present study than does $N_{4\sigma}$. The inversion of plate counts for methanol *versus* mobile phase found here using eqn. 4, however, as compared to actual peak shapes most likely derives from the increased emphasis of the b term in this equation

TABLE I

COLUMN EFFICIENCY/SAMPLE SOLVENT POLARITY DATA

Sample solvent	*Polarity index**	$N_{5\sigma}$	$N_{4\sigma}$	N_{SYS}
Mobile phase	8.2**	268	407	108
Methanol	5.1	91	410	122
Chloroform	4.1	193	328	218
2-Propanol	3.9	56	107	85
Butyl chloride	1.0	29	152	9
Hexane	0.1	178	254	132

* Ref. 31.

** $P_i \approx \varphi_1 P_1 + \varphi_2 P_2$, where φ_1 and φ_2 are volume fractions and P_1 and P_2 are polarity indices for water and methanol in the mobile phase respectively.

TABLE II

PEAK ASYMMETRY/SAMPLE SOLVENT POLARITY DATA

Sample solvent	*Polarity index**	*Symmetry factor (S) (eqn. 5)*	*Tailing factor (T) (eqn. 6)*	*Qualitative peak description***
Mobile phase (40% methanol)	8.2	2.25	1.62	a
60% Methanol	7.1	3.27	2.14	a
70% Methanol	6.6	1.58	1.29	a
80% Methanol	6.1	1.36	1.18	b
90% Methanol	5.6	1.06	1.03	b
100% Methanol	5.1	0.70	0.85	c
Chloroform	4.1	1.11	1.06	c
2-Propanol	3.9	0.51	0.75	b
Butyl chloride	1.0	4.09	2.54	d
Hexane	0.1	3.0	2.0	a

* Ref. 31.

** a, Single peak with tailing; b, single peak with fronting; c, split peak on fronting edge; d, split peak on tailing edge.

appearing as an inverse cubic power. The higher b value in the tailing peak (Fig. 1B) than in the split peak (Fig. 1A), gave the lower value of N_{SYS} for mobile phase as sample solvent than for methanol. The lack of relation between column efficiency calculated using either eqn. 1, 2 or 4 and sample solvent polarity over this broad range of solvents must be concluded, however.

Results found for peak symmetry and tailing factors calculated for the various sample solvents including the narrower range of polarity differences from 100% to 40% methanol (mobile phase) are shown in Table II. Approximate agreement is found between each of these measures for each sample solvent with values > 1.0 for tailing peaks and values < 1.0 for fronting peaks. A somewhat wider variation was found for the symmetry factor than for the tailing factor. While these calculations do not give a direct indication of peak splitting, they incorrectly show that the sample solvent closest to ideal is 90% methanol. This results from the fronting portion of the peak of approximately the same length as the tailing portion.

When $N_{5\sigma}$ calculations were made for various percentages of methanol in the sample solvent, results shown in Fig. 3 were obtained. The smooth transition from

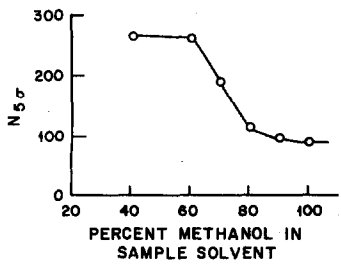


Fig. 3. Relationship between column efficiency (number of theoretical plates calculated as 5σ) and percent methanol in sample solvent for I on a β-cyclodextrin column.

split peak to a single peak is indicated as the sample solvent methanol content approached that of the mobile phase. This behavior is what could result from a kinetically controlled interaction in which a single rate of mass transfer between stationary phase and mobile phase is attained. Using methanol as sample solvent, a portion of I bound to the β-CD is solubilized and carried forth as an additional peak on the front edge of the main peak. This could result from a disruption of hydrogen bonds between I and hydroxyl groups at the mouths of the β-CD cavities when methanol is the sample solvent. It was not seen when methanol was sample solvent on the ODS-3 column because of a lower rate constant for mass transfer from stationary to mobile phase.

The question remains as to why no actual separation of enantiomers of I or II occurred on the β-CD stationary phase. According to the Dalgliesh three point theory, three bonds must be formed between the enantiomer and substrate to distinguish between (+) and (−) isomers[27]. In the case of the β-CD stationary phase whose retention mechanism was investigated by Hinze *et al.*[10], enantioselectivity results from the inclusion complexation plus two interactions with hydroxyl groups on the cyclodextrin cavity's rim. Little more is known as to exact molecular requirements for separation on a β-CD column although inclusion complexation by β-CD has previously been discussed[28–30]. From a literature review of the successful separations on this column, it is apparent that each enantiomer contains a naphthyl, indole or phenyl ring system at a distance of 1–3 atoms from the chiral center. In the present investigation the chiral carbon of the naphthyl ketone derivative (I) is five atoms away from the chiral center whereas the indole moiety of both I and II, although 1 atom away from the chiral carbon, does not fulfill the requirements for a chiral separation. It can be concluded that enantiomers must possess the correct functional groups which fulfill appropriate geometrical requirements for enantiomeric separation on- β-CD to occur. In addition, the proper choice of sample solvent must be made to avoid pseudochiral separations. The chromatographer should be aware of this possible obstacle to developing chiral separations.

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