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Chiral separation retention mechanisms in high-performance liquid chromatography using bare silica stationary phase and β-cyclodextrin as a mobile phase additive

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Abstract

This report describes a novel approach to direct chiral separations using bare silica stationary phase with an aqueous mobile phase containing sodium phosphate, β-cyclodextrin (β-CD), and 2-methyl-2-propanol. Two racemic cycloheptaindole derivatives were used as model compounds for the study. Both enantiomer pairs were resolved in a single chromatographic run. Analyte retention mechanisms were consistent with ion-exchange, β-CD inclusion, and hydrophobic modes of interaction. Adding triethylamine to the mobile phase improved peak symmetry and reduced retention. Consistent separations were obtained with different lots of β-CD and different analytical columns. The technique has a number of attractive features that may broaden the use of chiral mobile phase additives for direct enantioseparations.

1. Introduction

Chiral separations are a topic of great interest because of their importance in several fields, including biomedical research. High-performance liquid chromatography (HPLC) is the primary chiral separation technique used for pharmaceutical applications. Three approaches are generally used to achieve chiral separations by HPLC: indirect separations using chiral reagents to form permanent diastereomers, direct separations using a chiral stationary phase (CSP), and direct separations using chiral mobile phase additives [1]. Several reports demonstrate the utility of β-cyclodextrin (β-CD) as a chiral mobile phase additive (CMPA) for the direct separation of enantiomers using reversed-phase HPLC [2,3].

β-CD is a cyclic oligosaccharide composed of seven D-(+)-glucopyranose units joined by α-1–4 linkages. CDs are capable of forming inclusion complexes with other molecules. Enantiomers may show different inclusion complex stabilities based on differences in hydrophobic and hydrogen bonding interactions with the β-CD molecule.

Silica has been largely overlooked as a potential stationary phase for CMPA HPLC separations with β-CD. Gazdag et al. [4] noted the advantage of employing a cyanopropylsilica bonded phase for the enantioseparation of some model compounds with CD CMPA because of the low organic solvent concentration required to achieve reasonable retention times. Solubility of β-CD diminishes rapidly as the organic solvent

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concentration is increased. Gazdag et al. [5] suggested using silica stationary phase for non-polar molecules but no data were presented. Walhagen and Edholm [6] later used this approach to separate chlorthalidone and oxazepam enantiomers with a mobile phase of β-CD in ammonium acetate buffer.

The goal of our research was to investigate the chromatographic behavior of a system comprised of β-CD CMPA and bare silica stationary phase. A racemic, serotonin receptor agonist (DU 124884), and its potential N-desmethyl metabolite (KC 9048) were used as model compounds for the study. Both molecules contain one chiral center. Retention and enantioselectivity were characterized as a function of mobile phase conditions and column type.

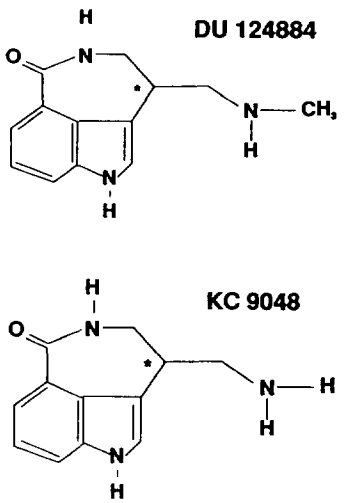

Fig. 1. Structures of model chiral amines DU 124884 (free base) and KC 9048.

2. Experimental

2.1. Materials

DU 124884, 99.9% purity, {(±)-3-methylaminomethyl - 3,4,5,6 - tetrahydro - 6 - oxo - 1H-azepino[5,4,3-*cd*]indole hydrochloride} and KC 9048, 95% purity, {(±)-3-aminomethyl-3,4,5,6-tetrahydro - 6 - oxo - 1H - azepino[5,4,3 - *cd*]indole} were synthesized by Solvay Pharma Deutschland Research Labs. (Hannover, Germany). Fig. 1 shows the structures of these compounds. β-CD, reagent and high-purity grades, was purchased from TCI Americas (Portland, OR, USA). The following high purity (≥ 99%) reagents were purchased from Aldrich (Milwaukee, WI, USA): 2-methyl-2-propanol, triethylamine, monobasic and dibasic sodium phosphate. Phosphoric acid, 85%, was also obtained from Aldrich. Deionized water was obtained by reverse osmosis using the Nanopure filtration system (Barnstead, Dubuque, IA, USA).

2.2. Standard solutions

Aqueous standard solutions of DU 124884 and KC 9048 used for chromatographic studies were prepared by serial dilution from a 1 mg/ml stock solution of racemic free base in water. Analytical standard concentrations were 100 and 10 μg/ml. The stock standard was stable for at least a month when stored at 4°C. Analytical standard solutions were prepared fresh weekly.

2.3. Chromatographic instrumentation and conditions

The HPLC apparatus consisted of a Rheodyne (Cotati, CA, USA) Model 7130 six-port injection valve with a 20-μl sample loop, a 232D pump, and a UV-100 variable-wavelength detector with deuterium source lamp (SSI, State College, PA, USA). A second system used for some studies was identical with the exception of an SSI Model UV 106 fixed-wavelength detector with a mercury source lamp. The separation was performed using a silica NewGuard cartridge guard column, 15 × 3.2 mm I.D., 7 μm particle size (Applied Biosystems, Foster City, CA, USA) and a Supelcosil LC-Si 250 × 4.6 mm I.D., 5 μm particle size silica analytical column (Supelco, Bellefonte, PA, USA). A Spherisorb silica 250 × 4.6 mm I.D., 5 μm particle size analytical column (MetaChem Technologies, Torrance, CA, USA) and a Zorbax Rx-SIL 150 × 4.6 mm I.D., 5 μm particle size silica

analytical column (MAC-MOD Analytical, Chadds Ford, PA, USA) were used in a manufacturer comparison study. A column water jacket and temperature-controlled circulating water bath were used to study temperature effects. Detector output was collected and analyzed using a personal computer equipped with VIS4 chromatography software (SSI).

The mobile phase composition is described in the figure legends and text of the Results and discussion section. All mobile phases were filtered through a 0.45-μm nylon 66 membrane filter before use. A flow-rate of 1.00 ml/min was used in all experiments. Unless indicated otherwise, work was conducted at an ambient temperature of approximately 21°C. The column was equilibrated with a minimum of 15–20 column volumes of mobile phase between composition changes. Absorbance was monitored at 231 nm with the variable-wavelength detector or 254 nm with the fixed-wavelength detector. The system void volume was calculated using the column porosity factor provided by the manufacturer. The accuracy of this estimate was confirmed by measuring the time to the first deflection in the baseline upon injecting water. Data represent the average of two or more injections where retention times agreed within 2%.

3. Results and discussion

3.1. Inclusion complex equilibria

According to Walhagen and Edholm [6], retention in systems containing β-CD as a CMPA can be described by the following relationship:

$$\frac{1}{k} = \frac{1}{k_0} + \frac{[CD]K_f}{k_0}$$

Thus, a plot of $1/k$ versus the cyclodextrin concentration will be a straight line through the origin, assuming that no other retention mechanism exists. The plot yields a slope of K_f/k_0, where K_f is the apparent formation constant for the inclusion complex and k_0 is the retention factor in the absence of CD. This treatment assumes that CD and complexed analyte are not retained by the stationary phase.

Studies to evaluate the influence of β-CD on retention were conducted using a mobile phase composition of 10 mM sodium phosphate buffer, pH 7, and 1% (w/v) 2-methyl-2-propanol (t-BuOH). Fig. 2 shows the effect of β-CD on retention and separation factor. The linear regression correlation coefficient for a plot of $1/k$ versus β-CD exceeded 0.993 for all four enantiomers. The following values for K_f (M^{-1}) were calculated from the plot: (−)-KC 9048 = 26, (+)-KC 9048 = 17, (−)-DU 124884 = 32, and (+)-DU 124884 = 21. Accordingly, the separation factor also varied as a function of β-CD concentration. The enantiomer pairs were unresolved at 0, 1 and 2 mM β-CD, but separation

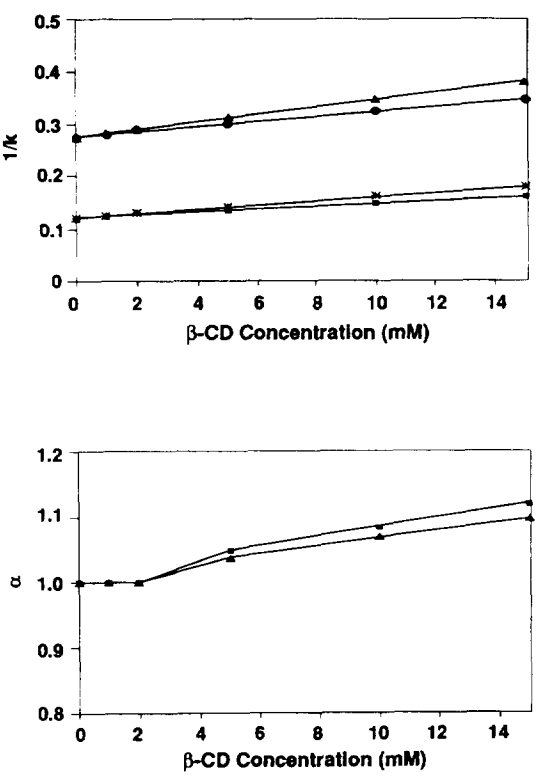

Fig. 2. Retention and separation as a function of β-CD concentration. Mobile phase: 10 mM sodium phosphate/1% t-BuOH, pH 7. Top: ▲ = (−)-KC 9048; ● = (+)-KC 9048; * = (−)-DU 124884; ■ = (+)-DU 124884. Bottom: ▲ = KC 9048; ■ = DU 124884.

factors increased as the β-CD concentration increased from 5 to 15 m*M* β-CD.

3.2. Ion-exchange equilibria

Retention of basic compounds on silica under "reversed-phase" conditions is a complex function of the pH, and the organic solvent and buffer concentrations [7]. Retention mechanisms are a combination of ion-exchange interactions with ionized silanol groups and hydrophobic interactions with siloxane groups on the silica surface [8].

Sodium ion concentration effects

The effect of buffer concentration on k and α for the two enantiomer pairs was evaluated from 16 to 160 m*M* sodium. The pH, β-CD concentration, and % t-BuOH were held constant at 7, 15 m*M* and 1%, respectively. The reciprocal retention factor increased non-linearly as a function of the sodium ion concentration. The separation factor was unaffected by sodium ion concentration.

pH effects

The silica surface has a pK_a of about 6.5 [8]. Thus, at pH 7 the surface is ionized and capable of cation-exchange retention. The effects of pH were studied from pH 3 to 7. The ionic strength was held constant ($\mu = 0.06$) while the pH was altered by the appropriate combination of three phosphate buffer solutions: H_3PO_4, NaH_2PO_4 and Na_2HPO_4. Fig. 3 shows the retention factor increased non-linearly as the pH was increased. The separation factors for DU 124884 remained unchanged as a function of pH. KC 9048 separation was optimal at pH 7 due to insufficient retention ($k < 2$) under more acidic conditions.

3.3. Alcohol modifier and hydrophobic retention

The elution order of DU 124884 and its N-desmethyl metabolite was consistent with the elution order expected for reversed-phase separations using alkyl bonded-phase columns. The alcohol modifier was necessary for inclusion

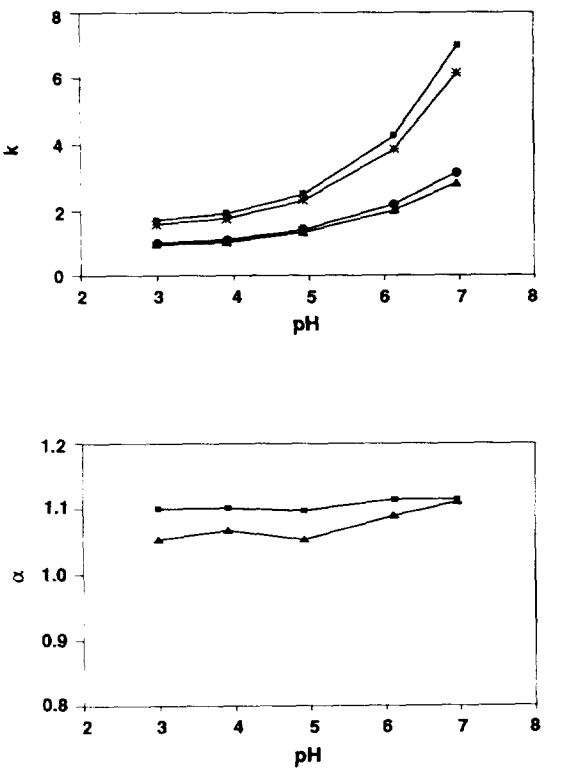

Fig. 3. Retention and separation as a function of mobile phase pH. Mobile phase: 10 m*M* sodium phosphate buffer/15 m*M* β-CD/1% t-BuOH. Symbols as in Fig. 2.

complex formation. Other workers have noted the ability of t-BuOH to stabilize analyte–β-CD complexes [3]. In the absence of t-BuOH, mobile phases of 10 m*M* sodium phosphate buffer (pH 7), and 10 m*M* sodium phosphate buffer (pH 7)/15 m*M* β-CD produced identical analyte retention factors.

Fig. 4 shows the influence of the organic modifier concentration on retention. The mobile phase composition was 10 m*M* sodium phosphate buffer, pH 7/15 m*M* β-CD. The reciprocal retention factor increased non-linearly as a function of the percentage t-BuOH from 0.5 to 2.5%. Peak splitting of KC 9048 occurred at 2.5% t-BuOH and retention factors were not calculable as a result of the distortion. The reason for the peak splitting is not clear. Separation factors did not change between 0.5 and 2.5% because t-BuOH was present in large

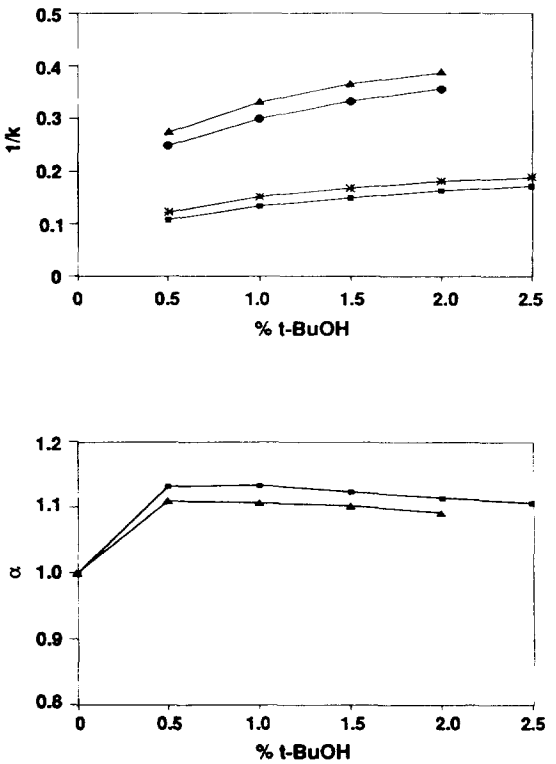

Fig. 4. Retention and separation as a function of percentage t-BuOH. Mobile phase: 10 m*M* sodium phosphate buffer/15 m*M* β-CD, pH 7. Symbols as in Fig. 2.

molar excess at all concentrations (0.5% = 67 m*M*).

3.4. Influence of amine modifier

Analyte peaks displayed poor symmetry with the three-component mobile phase. Organic amine modifiers are commonly used in reversed-phase separations of non-chiral amines to improve peak symmetry through hydrogen bonding, ionic interaction, or adsorption to the silica surface [8]. Fig. 5 shows the improvement in peak shape upon addition of 2 m*M* triethylamine (TEA) to the mobile phase. The retention factors diminished from 0.5 to 2 m*M* TEA, but were unchanged between 2 and 3 m*M* TEA. Separation factors were constant over the TEA concentration range studied.

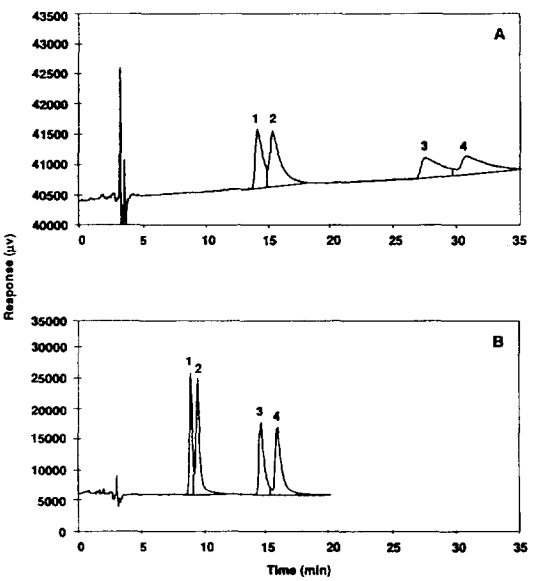

Fig. 5. Influence of triethylamine on the separation. (A) 10 m*M* sodium phosphate buffer/15 m*M* β-CD/1% t-BuOH, pH 7. (B) 10 m*M* sodium phosphate buffer/15 m*M* β-CD/1% t-BuOH/2 m*M* TEA, pH 7. Peaks: 1 = (−)-KC 9048; 2 = (+)-KC 9048; 3 = (−)-DU 124884; 4 = (+)-DU 124884.

3.5. Influence of the separation temperature

Fig. 6 shows a Van 't Hoff plot of log *k* versus $1/T$ using a mobile phase of 10 m*M* sodium phosphate buffer, pH 7/7.5 m*M* β-CD/1% t-

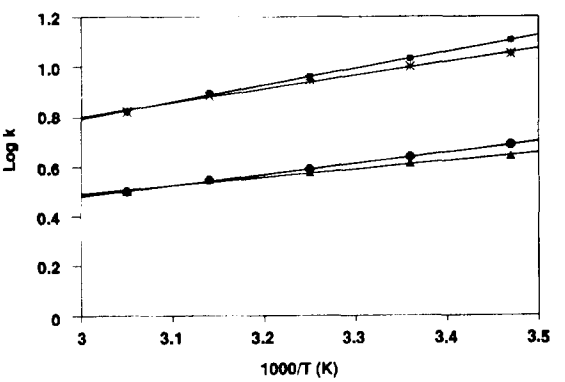

Fig. 6. Van 't Hoff plot of log *k* vs. $1000/T$ (K) using a mobile phase of 10 m*M* sodium phosphate, pH 7/7.5 m*M* β-CD/1% t-BuOH. ▲ = (−)-KC 9048; ● = (+)-KC 9048; * = (−)-DU 124884; ■ = (+)-DU 124884.

BuOH. The log-transformed data displayed excellent linearity ($r \geq 0.993$). Separation factors at 15°C using 7.5 m*M* β-CD were comparable to those obtained with 15 m*M* β-CD at ambient temperature (21°C). However, retention factors were greater at 15 vs. 21°C, and β-CD shows limited aqueous solubility at lower temperatures (11 m*M* at 15°C) [9]. Therefore, it is doubtful that any advantage would be gained by operating at subambient temperatures with β-CD CMPA.

3.6. System ruggedness

The ruggedness of the separation was evaluated by studying the influence of different lots and grades of β-CD on the separation. There was no change in retention or separation between lots or between grades of β-CD material. A comparison of different columns showed that retention factors differed but chiral resolution remained constant from column to column. Fig. 7 shows the results of a comparison of silica columns from different manufacturers. Once again, retention factors differed, but chiral separation remained constant. Retention factors can be easily optimized by changing the buffer concentration without deleterious effects on α. All columns provided adequate direct separations for both enantiomer pairs in less than 24 min. The column used for the retention mechanism studies was subjected to 500 injections over a 12-month period without an appreciable change in chromatographic performance.

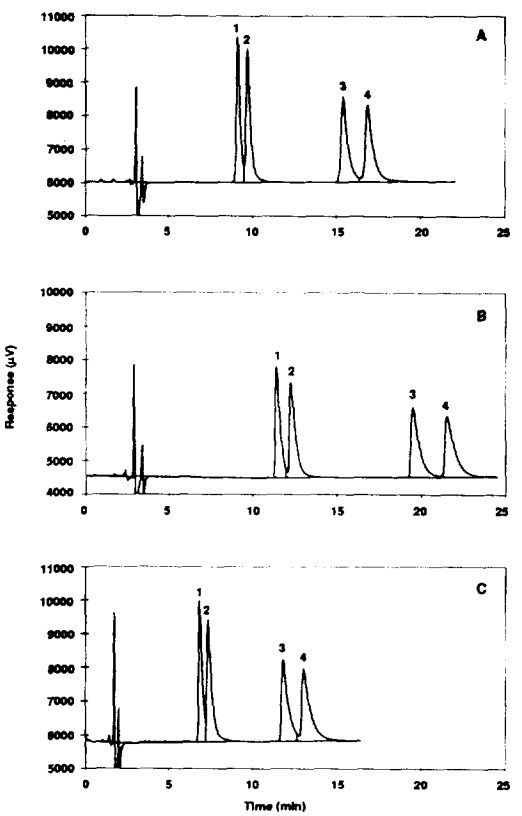

Fig. 7. Comparison of different manufacturers silica columns. (A) Supelcosil LC-Si, 250 × 4.6 mm; (B) Spherisorb SIL, 250 × 4.6 mm; (C) Zorbax Rx-SIL, 150 × 4.6 mm. Peaks: 1 = (−)-KC 9048; 2 = (+)-KC 9048; 3 = (−)-DU 124884; 4 = (+)-DU 124884.

4. Conclusions

This study demonstrates the utility of bare silica stationary phase with an aqueous–organic mobile phase containing β-CD for the enantioseparation of a pair of model pharmaceutical amines. The consistency of the separation between columns and between column manufacturers affords the chromatographer a great deal of flexibility and assurance that the method will not collapse with the next column purchase. An upcoming report will describe the application of this separation technique to the determination of DU 124884 in rat plasma.

Acknowledgements

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