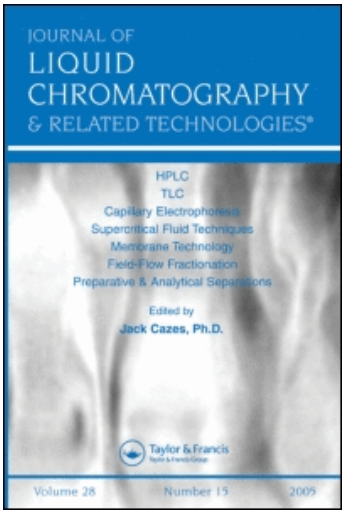

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Hara, Shoji , Ando, Tatsuhiko and Nakayama, Yoshiyuki(1989) 'Characterization of Column Packings in Normal-Phase Liquid Chromatography. I. Retention Behavior of Fat-Soluble Vitamins in Silica Gel-Binary Solvent Systems', Journal of Liquid Chromatography & Related Technologies, 12: 5, 729 — 738
To link to this Article: DOI: 10.1080/01483918908049203
URL: http://dx.doi.org/10.1080/01483918908049203

JOURNAL OF LIQUID CHROMATOGRAPHY, 12(5), 729-738 (1989)

CHARACTERIZATION OF COLUMN PACKINGS IN NORMAL-PHASE LIQUID CHROMATOGRAPHY. I. RETENTION BEHAVIOR OF FAT-SOLUBLE VITAMINS IN SILICA GEL-BINARY SOLVENT SYSTEMS

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ABSTRACT

To characterize packing columns in high performance liquid chromatography, the retention indices of ten fat-soluble vitamins were systematically measured using binary solvents each containing ethyl acetate, tetrahydrofuran and 2-propanol in n-hexane for silica gel chromatography. A linear relationship between the logarithm of the capacity ratio and that of the concentration of the polar solvents was confirmed. The retention sequence of the solutes was determined as follows: retinol > ergocalciferol = cholecalciferol > δ- > γ- > β- > α-tocopherol > menadione > phylloquinone. The retention behavior of retinal was similar to that of tocopherol derivatives, but varied depending on the polar solvent used. Such a retention sequence of fat-soluble vitamins may be explained on the basis of hydrogen bonding interactions between the active functional group on the solute molecules and silanol groups on the silica gel surface. Based on the adsorption selectivity given by the phase systems used, the resolution of each class of vitamins but not that of vitamin D homologues was successfully carried out.

INTRODUCTION

Various packing materials and columns have recently been developed for liquid chromatography (LC)[1,2]. In particular, the micro-porous bare silica gel and chemically-bonded silica gel packings have gained general acceptance owing to the high efficiency and resolution they provide. In reversed-phase mode separation, octadecylsilyl silica columns are used exclusively with aqueous methanol or aqueous acetonitrile as the mobile phase. In normal-phase mode separation, various columns and solvent mixtures as mobile phases can be used. Still, general guidelines that would facilitate in a practical way the selection of a column and optimization of a mobile phase for given solute samples are not available. In consideration of this, the characterization of packing columns should thus be carried out with respect to retentivity and selectivity using organic solvents as mobile phases.

To characterize LC packings, the accumulation and integration of the retention data of lipophilic substances have already been carried out using typical columns and mobile phase solvent systems at our laboratory[3-9]. The characterization of chemically bonded silica gel columns is possible by a comparison of their retention data with those obtained using a bare silica gel column as the standard. Thus, basic retention indices using silica gel-binary mobile phases were systematically measured in the present study as the first of a series of studies on ten biologically active fat-soluble vitamins. The results of a comparative study, the second of the series, using chemically bonded silica columns are presented in the following.

In normal-phase LC, peak shape non-ideality is often observed and it lessens the degree of separation efficiency. Peak sharpness and asymmetry given by fat-soluble viatamins used as solutes on silica gel-binary solvents were thus measured to evaluate the resolution functions of normal-phase LC systems.

EXPERIMENTAL

LC System

The instruments used were an LC system (LC-5A), UV-detector (SPD-2A) and a data processor (CR-2A, Shimadzu, Kyoto). Conventional columns packed with silica gel (250 x 4.6 mm, Zorbax, Du Pont, Wilmington, DE) were used.

Samples and Reagents

All trans retinol (1), retinal (2), ergocalciferol (3), cholecalciferol (4) and phylloquinone (10) were obtained from Sigma, St. Louis, MO. α- (8), β- (7), γ- (6), δ-Tocopherol (5) and menadione (9) were obtained from Wako Pure Chemicals, Osaka. Sample purity was checked by thin-layer chromatography (TLC) using a silica gel, amino- and cyano-type high performance TLC-plates obtained from E. Merck, Darmstadt. All solvents used were of analytical grade (Wako Pure Chemicals).

Procedure

Chromatographic runs were conducted at a constant flow rate of 2 ml/min and constant temperature of 20 C using an electrically controlled column-oven. The detector was operated at 254 nm and 292 nm. One to five milligram samples were dissolved in 10 ml of n-hexane and 5 μg of each sample solute were injected onto the column.

Retention data were obtained and peak widths determined so as to calculate the capacity ratios of solutes and evaluate peak sharpness and asymmetry [10].

The capacity ratio, k' was calculated from,

$$k' = (t_R - t_0)/t_0$$

where t_R, t_0 are the retention time and dead time, respectively. The latter was measured using benzene as the solute retained the least.

RESULTS AND DISCUSSION

1. Capacity Ratio vs. Solvent Composition

The solutes selected for determining retention characteristics were vitamin A, D, E, K homologues (Figure 1). All these molecules

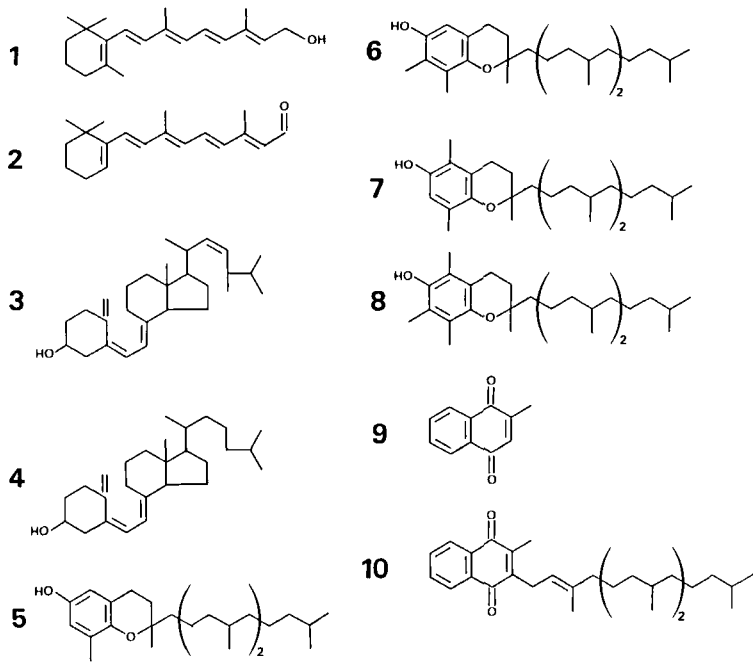

Figure 1. Structural Formulas of the Fat-Soluble Vitamins Examined.

1 = retinol, 2 = retinal, 3 = ergocalciferol, 4 = cholecalciferol, 5 = δ-tocopherol, 6 = γ-tocopherol, 7 = β-tocopherol, 8 = α-tocopherol, 9 = menadione, 10 = phylloquinone.

contain lipophilic bodies and one or two hydrophilic functional groups whose roles are relatd to the silanol groups of the silica gel surface. Polar solvent molecules in the binary mobile phase function as hydrogen bonding acceptors or donors, and should thus be in competition with solute molecules for interacting with silanol groups. By such a model involved in the competition of solute molecules with those of the solvent on a silica gel surface, the retention behavior of solutes can best be explained.

Based on this mechanistically constructed adsorption-desorption model, a mathematical relation between the capacity ratio and the solvent composition[11,12] was deduced and experimentally verified for silica gel-binary solvent systems in normal-phase LC as follows[4-7]:

$$\log k' = c - n \log X_s \qquad (1)$$

where k' and X_s are the capacity ratio and concentration, respectively, of the polar solvent in the binary system and c and n are constants. To obtain systematic retention data on the ten vitamins, the capacity ratios were estimated at various concentrations of the polar solvents in the n-hexane-binaries and their correlations with solvent compositions were determined. The polar solvents used were ethyl acetate (B1) and tetrahydrofuran (B2) as basic solvents and 2-propanol (AB) as the acidic-basic solvent. The experimental data obtained are shown in Figures 2-4. A linear relation between the logarithm of the capacity ratio and the logarithm of the concentration of B and AB solvents was confirmed and the correlation coefficient value was determined as 0.98.

2. Molecular Structure and Retentivity of the Solutes

In the three mobile phase systems used, retinol (1) gave the highest retention value (Fig. 2-4). The hydroxyl group in this compound is considered active for being adsorbed onto silanol groups present on the silica gel surface. Ergocalciferol (3) and cholecalciferol (4) also have hydroxyl groups, but their adsorption activity of this type was found to be less than that of retinol. The differences in adsorption activity can be explained on the basis of the steric effects and nature of the hydroxyl groups in both classes of solutes. Adsorptivity of the secondary alcohol function present in vitamin D homologues is usually less than that of the primary alcohol function in retinol. The separation of ergocalciferol and cholecalciferol was difficult owing to their structural similarity. Indeed, ergocalciferol is merely a methyl homologue derived from cholecalciferol.

The adsorption activity of the formyl group in retinal (2) is much weaker than that of the hydroxyl group and thus, the separation of vitamin A homologues can be affected much more easily than that of viatmin D homologues.

The adsorption of vitamin E homologues (5-8) is weaker than vitamin A and D derivatives. The phenolic hydroxyl group present in tocopherol derivatives may possibly contribute to hydrogen bonding association with silanol groups on the silica gel surface. The adsorption selectivity given by the three mobile phase solvents used is gen-

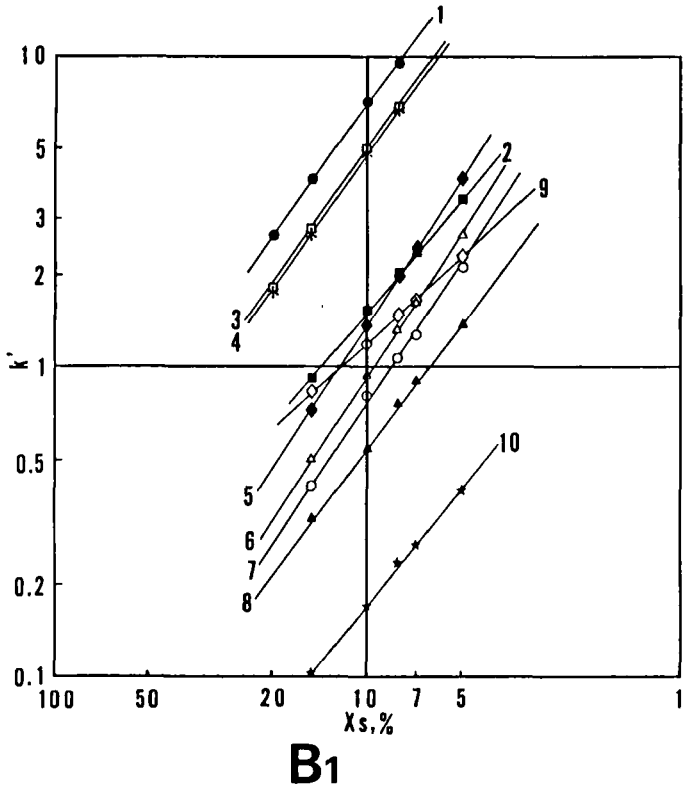

Figure 2. Logarithm of the Capacity Ratio on a Silica Gel Column as a Function of That of Ethyl Acetate (B1) Composition in n-Hexane.

Samples are the same as in Figure 1.

erally moderate and suited for the resolution of vitamin E homologues. An example of this resolution is shown in Figure 5. The retention sequence owes to the steric hindrance toward the phenolic hydroxyl groups in the vitamin E derivatives. A single methyl group located at the meta position of a hydroxyl function should have the least steric effect on the adsorption activity of the solute, and accordingly, the adsorptivity of δ-tocopherol (5) was greatest among the four homologues. Two methyl groups located at the ortho and meta positions of

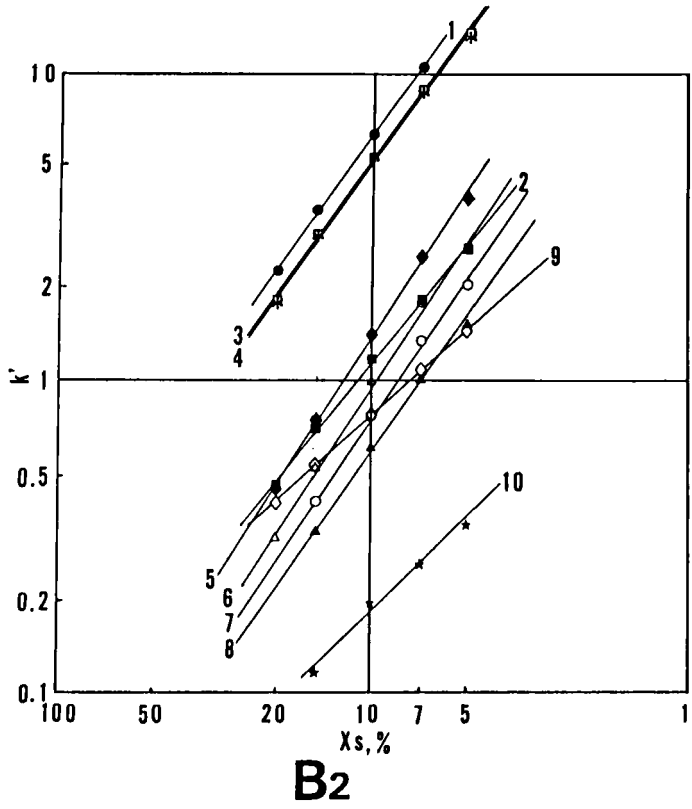

Figure 3. Logarithm of the Capacity Ratio on a Silica Gel Column as a Function of That of Tetrahydrofuran (B2) Composition in n-Hexane.

Samples are the same as in Figure 1.

the hydroxyl group in β- (7) and γ-tocopherol (6) showed a decrease in adsorption activity. The steric hindrance associated with these groups in β- and γ-tocopherol was found to differ and the two compounds could thus be separated by any one of the three solvent systems. Two of the three methyl groups neighboring the hydroxyl group on the benzene ring caused the most decrease in the retention of α-tocopherol (8).

The vitamin K homologues (9,10) showed the weakest adsorptivity. The retentivity of phylloquinone (10) was less than that of menadione

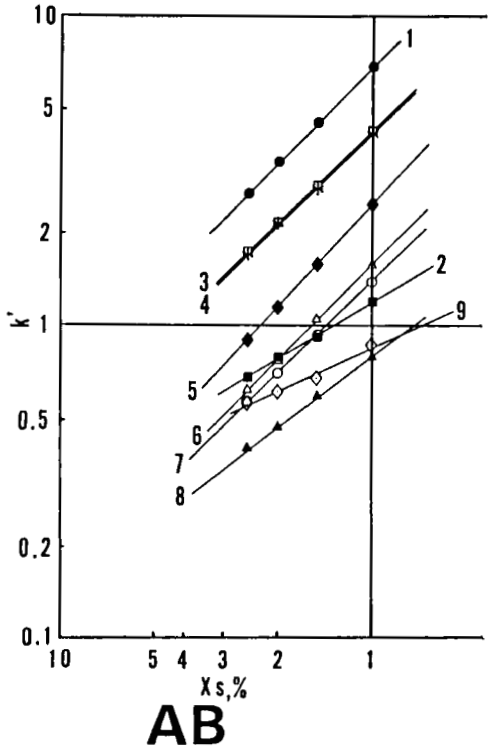

Figure 4. Logarithm of the Capacity Ratio on a Silica Gel Column as a Function of That of 2-Propanol (AB) Composition in n-Hexane.

Samples are the same as in Figure 1.

(9). The steric hindrance of the long alkenyl chain introduced into the quinone nucleus was remarkable, as evident from the retention behavior of these two solutes (Figures 2-4).

3. Roles of Polar Solvents in Elution Strength, Selectivity and Column Efficiency

The retention behavior of the solutes for two binary mobile phase systems made by ethyl acetate (B1) or tetrahydrofuran (B2) as the polar component was essentially the same, as evident from Figures 2 and 3. The elution strength of 2-propanol (AB) in n-hexane, in contrast,

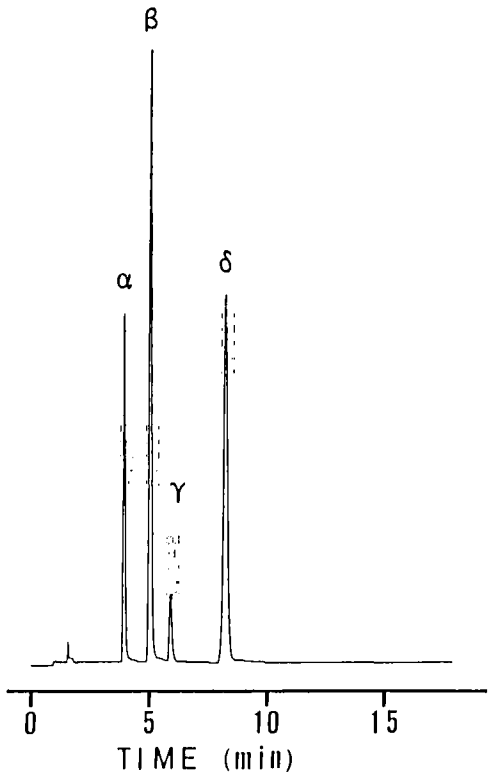

Figure 5. Chromatographic Resolution of Vitamin E Homologues on Silica Gel Using Ethyl Acetate in n-Hexane (5% v/v) as the Mobile Phase.

greatly exceeded that of the former two solvent systems. The selectivity of the AB solvent as measured by intercept difference (Δlog k') was less than that of the two B solvents. In this aspect, B solvent systems should be used to obtain higher resolution of a sample mixture containing fat-soluble vitamins.

However, in the case of the resolution functions of systems measured by peak sharpness and peak asymmetry using various solvent systems, the situation is different from that of elution selectivity. The peak sharpness given by three polar solvents in n-hexane binaries was basically the same while the peak asymmetry given by the AB solvent

system was much less than that of either of the two B solvent systems. The 2-propanol-binary system is thus concluded superior for affecting the resolution of fat-soluble vitamins using a silica gel column. A comparison is to be made of the experimental data on peak sharpness (Q) and the asymmetry factor (As) on a silica gel column with those obtained using chemically bonded columns in our next paper.

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