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ROLE OF MOBILE PHASE COMPONENTS FOR MULTI-FUNCTIONAL SOLUTES IN SILICA GEL LIQUID–SOLID CHROMATOGRAPHY

RESOLUTION OF PROTECTED DEOXYRIBONUCLEOTIDE DERIVATIVES FOR OLIGOMER SYNTHESIS

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SUMMARY

To improve the resolution of a given sample mixture in liquid–solid chromatography, the characteristics of various components in the mobile phase were determined with a mixture of closely related compounds associated with DNA oligomer synthesis. Water (S_0) was added so as to reduce peak tailing efficiently, but in amounts that would not significantly diminish retentivity in the silica gel column. The role of the stronger solvents, such as 1-propanol (S_1) and dioxane (S_2), in *n*-hexane (W) as diluent was quantitatively evaluated to control the retentivity. A linear correlation was found between the logarithms of the capacity ratios and molar concentration of the stronger component in the ternary solvent mobile phase. The selectivity of two stronger solvents was incorporated into the phase system to increase the resolution function for four given samples. A quaternary solvent system (W + S_1 + S_2 + S_0) was prepared by integrating the characteristics of all the components, as determined by regression analysis. The multi-component phase system led to a high degree of resolution. This effective procedure was found suitable for analytical and preparative purposes in the resolution of various complex mixtures.

INTRODUCTION

In organic synthesis and natural product chemistry, silica gel liquid–solid chromatography (LSC) has been highly preferred as a means of sample preparation. Since organic solvents as eluents can be easily removed, LSC is the most suitable technique for preparative purposes. It is also capable of resolving a mixture containing closely related organic solutes having different functional groups and steric configurations[1]. By selecting and blending various solvents for a mobile phase, a wide range of retentivity and selectivity is possible and a multi-component phase system can be adjusted precisely for high resolution of a given sample mixture[2]. However, there are several problems involved in LSC separation, such as the deterioration of the resolution function as a result of peak tailing.

Various explanations have been given for peak tailing[3]. A silica gel surface is

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known to have microscopic heterogenity regardless of its chemical purity and to contain energetically heterogeneous active sites. Since a solute is more adsorbed by stronger active sites than normal active sites on the silica gel surface, the process of adsorption–desorption equilibrium is necessarily slow, possibly giving rise to peak tailing. The multi-functional solutes used in organic synthesis are generally of high-polar character and thus are closely related to the induction of peak tailing and diminution of resolution.

Recently, the importance of water for the stationary phase in LSC has been suggested[4] and the effects of the addition of water to the mobile phase on peak shape have been reported[5,6]. Since water is trapped tightly in the stronger adsorption sites, it should certainly be suitable as a mobile phase component for tailing reduction. Water is generally considered to decrease solute retention in proportion to the extent to which the activity of the silica gel surface is diminished. However, it should be noted that the addition of water to the mobile phase does not always decrease solute retention[7]. If the addition of water to the mobile phase leads to a reduction in peak tailing without excessive diminution and extreme alteration in retentivity for a sample mixture, it may be used as an ideal tail reducer in the resolution of a multi-functional solute mixture.

With regard to the preparation of a solvent system for LSC, the retentivity and selectivity of a given sample mixture can be controlled through the use of various stronger solvents. If water is initially added to reduce tailing, followed by optimizing a multi-component phase system by using stronger solvents of suitable retentivity and high selectivity with a diluent, a high degree of separation can be expected.

For the actual realization of such a system, the effects of the addition of water on tailing reduction were examined. Control of retentivity and selectivity was carefully investigated with a group of samples having multi-functional groups in their molecules. The samples were the intermediates used in connection with a synthetic study of DNA oligomers.

EXPERIMENTAL

Samples

Solutes examined are synthetic intermediates for DNA oligomer preparation. Sample a: 5′-di-4-methoxytritylthymidine, b_1 and b_2: diastereomers of 2-cyanoethyl-4-chlorophenyl-5′-di-4-methoxytritylthymidine-3′-phosphate, c: 4-chlorophenyl-3′,3′-bis(5′-di-4-methoxytritylthymidine)phosphate. These protected deoxyribonucleosides and deoxyribonuclotide derivatives were prepared in our laboratory from deoxyribonucleosides by the triester method[8].

Reagents

The starting material, reagents for protection and coupling reactions and solvents were purchased from Wako (Osaka, Japan) and Aldrich (Milwaukee, WI, U.S.A.). The reaction products were purified with silica gel LSC using benzene–methanol as the eluent and the purity was determined by thin-layer chromatography and nuclear magnetic resonance spectrometry.

Apparatus

In a Model 5021 chromatograph (Varian, Palo Alto, CA, U.S.A.), the mobile phase solvents were pumped into a column. The sample solution into a 10-μl loop was injected. The column was 25 × 4.6 mm I.D. and packed with 1.9 g of spherical porous silica gel with 10-μm average particle size, 95 Å average pore diameter, and about 380 m^2/g specific surface area (Kusano Scientific, Tokyo, Japan). A UV detector SPD-1 (Shimadzu, Kyoto, Japan) and a chromatogram processor SIC Model 8000A (System Instruments, Tokyo, Japan) were used.

Procedure

The samples were dissolved in an eluent or chloroform. An ambient temperature of 24 ± 1°C was maintained during chromatography. Prior to analysis, each eluent having a volume 30 times the void volume of the column was passed through it at 40°C. The flow-rate of the eluent was 1 ml/min. The absorption of light (wavelength, 280 nm) by the eluate was recorded. The mean values of the capacity ratio ($k' = (t_R - t_0)/t_0$, t_0 = time equivalent to the column void volume), asymmetry factor, *As*, and relative peak sharpness, *Q*, were obtained from three calculations after determining the equilibrium of the phase system. The t_0 was determined by measuring the time from the injection of *n*-hexane to the first baseline disturbance.

RESULTS AND DISCUSSION

To determine the role of a component in the multi-solvent phase system, synthetic products related to the oligodeoxyribonucleotide preparation were selected as model samples. These products contained closely related multi-functional solutes, such as a protected nucloside (a), two diastereomeric phosphorylated nucleosides (b_1 and b_2) and a dimeric nucleotide (c) derived from thymidine. Compound a was the starting material for the synthesis, compounds b_1 and b_2 the product isomers and compound c the side-product to be eliminated. The deprotection reaction of compounds (b_1 and b_2 leads to a single product and, thus, it was quite unnecessary to separate them from each other. In recent years, deoxyribonucleotide oligomer synthesis has been the object of study in genetic engineering, an important field of biotechnology. A fast and reliable procedure for the purification of synthetic intermediates is particularly desirable for an efficient liquid phase bulk preparation of DNA oligomers. We have also been engaged in this area of research, and consequently we investigated the optimization of a phase system to resolve such synthetic intermediates.

A mobile phase was prepared basically by a diluent (W) and stronger solvent (S). To suppress tailing associated with multi-functional solutes having polar character in LSC, water (S_0) was added as a modifier. Various procedures for peak shape estimation have been proposed. In this study, relative sharpness[9], *Q*, and asymmetry factor[5], *As*, were employed as parameters. These parameters are defined as follows: $Q = t_R/t_W$, where t_R and t_W are the retention time and peak width, respectively, and $As = {}_hW_r/{}_hW_f$, where ${}_hW_r$ and ${}_hW_f$ are backward- and forward-half peak width at the 10% peak height, respectively. Increasing *Q* and decreasing *As* connote improvement in peak shape.

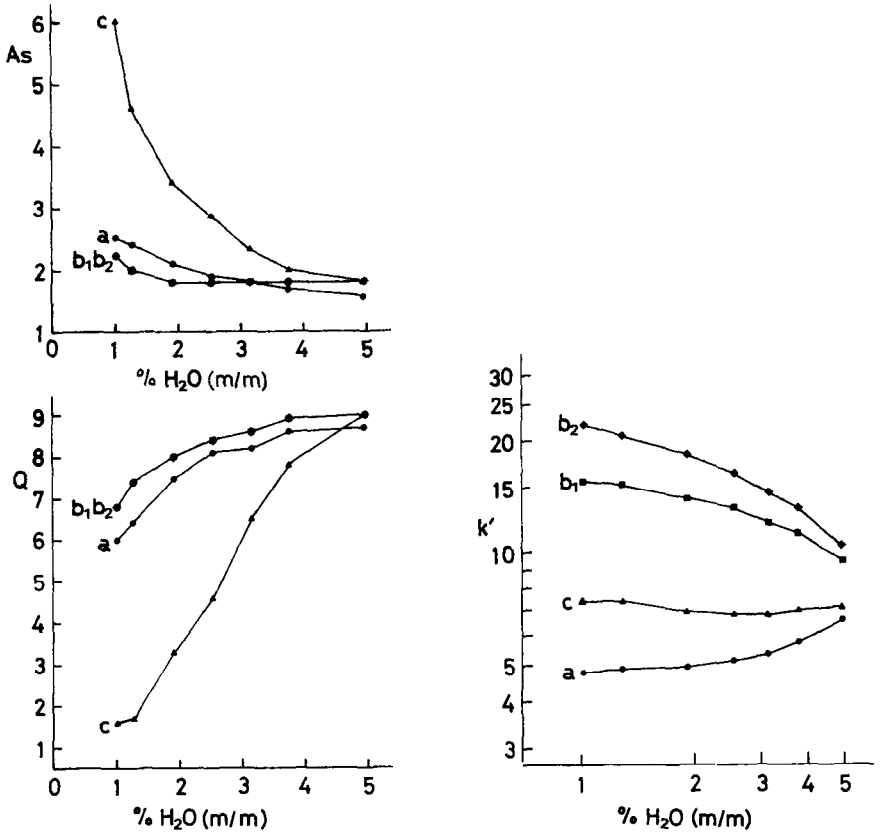

Fig. 1. Effects of water in the eluent on the asymmetry factor, relative sharpness of tailed peak and capacity ratio. Samples: a = 5'-di-4-methoxytritylthymidine, b_1 and b_2 = diastereoisomers of 2-cyanoethyl-4-chlorophenyl-5'-di-4-methoxytritylthymidine-3'-phosphate, c = 4-chlorophenyl-3',3'-bis(5'-di-4-methoxytritylthymidine)phosphate. *As* was determined by the formula: $As = {}_bW_r/{}_bW_f$ where ${}_bW_r$ and ${}_bW_f$ are the half peak width on the time scale at the 1/10 peak height for the backward and forward directions, respectively. *Q* was determined by the formula: $Q = t_R/t_W$ where t_R and t_W are the retention time and peak width, respectively. Eluent: *n*-hexane (W)–1-propanol (S_1) (85:15, v/v) + water (S_0). Adsorbent: silica gel.

Effects of water on peak shape in the binary solvent mobile phase

A solvent miscible with water was used in preparing a water-containing homogenous solvent system with *n*-hexane (W) as diluent. Because of its high polarity, such a solvent would function as a stronger component (S). Alcohol analogues, tetrahydrofuran and dioxane were tested as S components. Various binary solvent mixtures (W + S) were prepared that would afford the same capacity ratio of approximately 10 towards the most strongly retained sample. Two parameters for peak shape estimation and the retention values of the protected nucleoside derivatives were determined by varying the amount of water added to the binary solvent mixture. Experimental results obtained using 1-propanol S_1) as a stronger component are illustrated in Fig. 1. In the figure, it is evident that *As* decreases and *Q* increases, and thus peak tailing is reduced, by increasing water content. A decrease in retention,

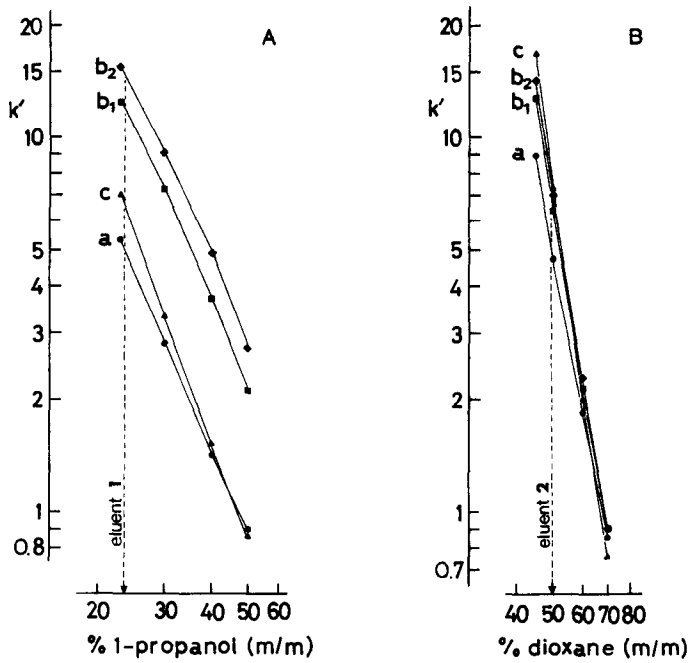

Fig. 2. Correlation between logarithms of capacity ratio and molar concentration of the stronger solvent in the eluent. Samples and adsorbent as in Fig. 1 and the water content in each solvent system is 3% (mol/mol).

particularly those of compounds b_1 and b_2 was observed with an increase in water content. However, the deviation in retention indices was rather small for all samples, especially at a water content of less than 3%. Therefore, the S_0 content was set at 3% in later experiments.

Retention control by stronger components in the ternary solvents

To control the retentivity of solutes and to find a suitable stronger component for optimum resolution, various S solvents were examined by mixing them with a diluent. Based on the data above, water was added to the diluent, *n*-hexane, to a 3% concentration. The retention behaviour of the protected nucleoside derivatives was determined using 1-propanol (S_1) or dioxane (S_2) as the stronger component in a ternary solvent system. The data are illustrated in Fig. 2A and B.

Linear correlation between the logarithms of the capacity ratios and molar concentration of the S component has been shown experimentally for binary solvent mobile phases and ternary systems as well, using various solutes in LSC[10–13]. Such a relationship was also confirmed in this paper for the ternary solvent system containing W, S and S_0 components when the S_0 fraction was constant. Water and the stronger solvent were responsible for the tail reduction and retention control, respectively.

Selectivity control by blending two stronger components

Let us now consider elution selectivity. The retention order of the solutes was

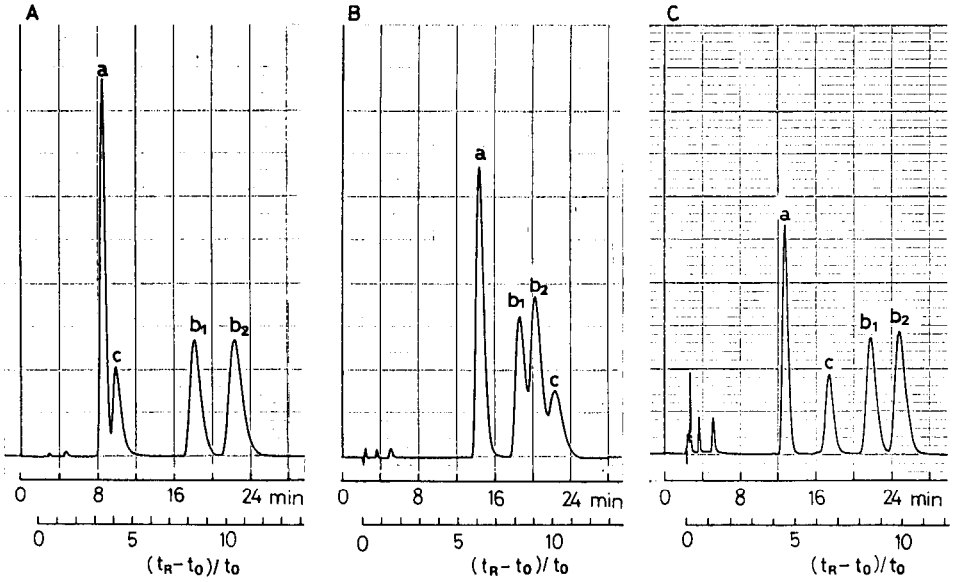

Fig. 3. Chromatograms of synthetic intermediates for oligodeoxyribonucleotide preparation using multi-component solvent systems. Eluents: A, *n*-hexane (W)–1-propanol (S_1)–water (S_0) (69:28:3, mol/mol), B, W–dioxane (S_2)–S_0 (49:48:3, mol/mol), C, W–S_1–S_2–S_0 [eluent 1–eluent 2 (76:24, mol/mol) as in text]. Samples and adsorbent are the same as in Fig. 1.

found to be as follows: a < c < b_1 < b_2 for the system containing less than 40% of the stronger component, S_1. Similar retention behaviour was shown for the system containing other alcohols and tetrahydrofuran as S components. With the S_2-containing solvent system (S_2 content < 50%), the order of c and the pair of b was reversed, causing the order to become a < b_1 < b_2 < c. Chromatograms obtained using S_1 and S_2 as stronger components are shown in Fig. 3A and B. In these chromatograms, the upper limit of the capacity ratio for the solute mixture was set at approximately 10.

Sample c was a by-product of the synthetic reaction, and thus its elimination from other samples was desirable. In the former system, recovery of the starting material a was hindered by the side product c and in the latter system, the b_1 and b_2 fractions were contaminated by compound c. No phase system was found capable of removing sample c from the other samples.

To solve this problem, retention control by blending two S components of different selectivity was considered. An increase in the retention of sample c relative to a, b_1 and b_2 by adding S_2 to S_1 to improve the resolution of samples a and c seems possible, since S_2 has a lower solvent strength toward sample c than S_1. Thus the effects of mixing S_1- and S_2-containing solvent systems on retention were examined experimentally.

At first, two solvent systems having a similar eluotropic property were selected to obtain standard data. The concentration of a solvent S that would give a capacity ratio of approximately 5 was determined for sample a on the basis of the data in Fig. 2A and B. At the equi-eluotropic concentrations (23.5% for S_1 and 50% for S_2 for

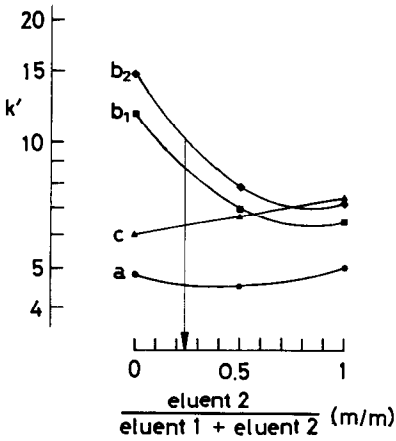

Fig. 4. Concept underlying optimization of the quaternary solvent system using regression analysis. Eluent 1 = *n*-hexane (W)–1-propanol (S_1)–water (S_0) (73.5:23.5:3, mol/mol), Eluent 2 = W–dioxane (S_2)–S_0 (47:50:3, mol/mol). Samples and adsorbent as in Fig. 1.

eluents 1 and 2, respectively) thus determined, retention indices of four samples for eluents 1 and 2 were experimentally confirmed. The data are illustrated in Fig. 4.

In order for samples b_1, b_2 and c to be of medium strength, a quaternary solvent system was prepared by adding eluent 2 to eluent 1 (1:1, mol/mol). For this solvent mixture retention values of the sample mixture were experimentally derived. These data appear in the middle of Fig. 4.

These data show that a certain approximation of retention behaviour may be possible at any composition of the phase system. As a result, quadratic regression analysis of log k' *vs.* fraction of eluent 2 in the quaternary system was carried out by computer. The regression curves for the quaternary solvent system, consisting of eluents 1 and 2 are shown in Fig. 4. A suitable composition of the two solvent systems in the quaternary solvent system can be directly predicted so that a maximum separation factor can be obtained for the sample pairs a, c and c, b according to computer simulation. The retentivity of a solute mixture was thus again experimentally measured using a system containing 76 mol/mol% of eluent 1 and 24 mol/mol% of eluent 2. The chromatogram obtained is illustrated in Fig. 3C. The retention values of four experimentally determined solutes were found to be approximately the same as those estimated. The percent error between these values was less than 5%. It is now possible to recover the starting material, a, first, to eliminate the side product c, and finally to fractionate the target products, b_1 and b_2.

This optimized solvent system was successfully applied to a large scale preparation of DNA oligomers. The procedure for the systematic design of multi-component mobile phase was employed for separating other families of compounds as well.

CONCLUSIONS

The role of the mobile phase components was determined using four synthetic intermediates for DNA synthesis. It was confirmed that peak tailing associated with

a multi-functional solute could be reduced by the addition of water without excessive alteration in retentivity. Solute retention could be continuously controlled by a stronger solvent. Selectivity control was possible by the proper choice and blending of two stronger solvents. On this manner an optimum system was prepared directly. A systematic procedure was drawn up for the design of a quaternary solvent system in the LSC separation of multi-functional solutes.

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