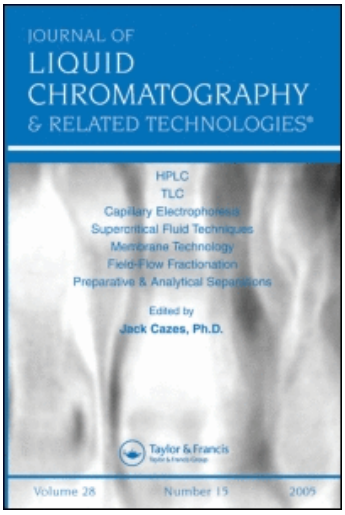

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DESIGN OF BINARY SOLVENT SYSTEMS FOR SEPARATION OF PROTECTED OLIGO-PEPTIDES IN SILICA GEL LIQUID CHROMATOGRAPHY

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ABSTRACT

In order to adjust the binary mobile phase for the separation of protected oligo-peptides, a systematic investigation of the retention behaviour on silica gel columns was carried out using seven peptide derivatives. Quantitative correlation between the capacity ratios of solvent systems using various concentrations of the stronger eluent was determined. The strengths and selectivities of fourteen binary systems were discussed on the basis of their retention indices. The design of equi-eluotropic binary systems containing these stronger components is presented.

INTRODUCTION

Detection of impurities in synthetic products and purification of lipophilic intermediates in synthesis have become an inevitable requirement in modern peptide chemistry. Liquid-solid chromatography (LSC) is generally accepted as being the most powerful tool for such purposes. A large amount of data has been accumulated concerning the separation of protected oligo-peptides; however, very limited attention has been directed toward systematic studies concerning retention behaviour related to the chemical structure of the solutes and optimization of the chromatographic parameters (1).

In LSC separation of lipophilic peptide derivatives, silica gel-binary solvent systems have been commonly applied. In the

meantime, a systematic procedure for designing binary mobile phases for use on silica gel columns has been developed by one of the authors (Hara) (2-7). In the present study, this procedure has been applied to the analytical study of protected oligo-peptides. Retention behaviours of seven synthetic intermediates were quantitatively determined by using systematically prepared binary mobile phases of various compositions. Solvent strength and selectivity of the binary systems in the separation of protected peptides were discussed on the basis of retention indices.

EXPERIMENTAL

Samples

Protected peptide derivatives were prepared by standard procedures from commercially available α-amino acids.

Columns

The silica gel packing, Wakogel LC-10H was irregularly shaped with a mean particle size of 10 μm and a pore diameter of 70 Å (Wako Pure Chemicals, Osaka). A silica gel slurry in chloroform/carbon tetrachloride/dioxan (2 : 1 : 2 v/v) was packed into a glass tube, 4 mm i.d. x 20 cm, CIG column system (2) (Kusano Scientific Co., Tokyo). The number of theoretical plates was 3000 per 20 cm using diethyl phthalate as the solute and n-hexane-ethyl acetate (9 : 1 v/v) as the solvent.

Chromatography

A KP-9H reciprocating pump (Kusano) and an R 401 refractive index detector (Waters Associates, Milford, Mass.) were linked to the on-column septum injector and the column end. Flow rate was 1 ml/min. Dead time was measured using cyclohexane as a sample. 5 mg samples were dissolved in 0.1 ml of the mobile phase solvent. When the sample compound was not soluble in the mobile phase solvent, it was dissolved in dichloromethane. A volume of 5 μl of the sample solution was injected into the column. Chromatography was performed at ambient temperature (ca. 15°C) and solvents were equilibrated

with ambient moisture prior to use. The capacity ratio k' was calculated according to the formula: $k' = t_s/t_m$, where t_s is adjusted retention time and t_m is dead time.

RESULTS AND DISCUSSION

The amide bond is present in the peptide structure and has polar characteristics that allow it to serve as either a donor or acceptor in hydrogen bonding interactions. The peptide bond may be repeated allowing chain elongation. As the degree of amide bonding in the solute increases, so does its polarity. Protecting groups having a lipophilic character decrease the retention of the solute molecules; however, the separation of a large range of peptides differing in degree of the oligomerization still requires a number of mobile phases covering a wide range of polarity.

Binary solvent systems composed of a diluent and a stronger eluent (S) should serve as a promising means of adjusting the retentions of protected peptides having various polarities. In preceding papers (2-4), a procedure for the systematic preparation and optimization of binary solvents was proposed. Such a process has been applied in the investigation described here as an improved means of peptide analysis.

It has been observed that hydrogen bonding is commonly involved in adsorption-desorption equilibrium as a leading interaction between the solute and the solvent molecules at the active sites of the adsorbent surface. Based on such an association phenomenon, the solvents have been classified according to diluent type: O, P and N and stronger eluent type: B and AB (1-5).

Four diluents: n-hexane (O), benzene (P), dichloromethane (N_1), chloroform (N_2) and eight stronger eluents: diethyl ether (B_1), ethyl acetate (B_2), acetone (B_3), tetrahydrofuran (B_4), dioxan (B_5), acetonitrile (B_6) and 2-propanol (AB) were employed for preparing binary mobile phases. The abbreviations for the solvents used in this paper were discribed in preceding papers (2-5). Binary solvent systems O + B_2, B_3, B_4, B_5 and AB were prepared and their capacity ratios on silica gel columns were determined using various concen-

1. Z-PHE-PHE-OBu^{T}
2. Z-PHE-PHE-OET
3. Z-PRO-PHE-OET
4. Z-TYR-IIE-OET
5. BOC-HIS(BZL)-PRO-PHE-OET
6. BOC-HIS(BZL)-LEU-OBZL(NO_2)
7. Z-VAL-TYR-IIE-OET

FIGURE 1. Structural formulas of the protected peptides investigated.

trations of the S component. The eluting strength of the O + B_1 system, however, was so small that the system could not be tested. Since solubility is an important factor in preparative separation, several of the more soluble P + B and N + B systems were additionally tested on the protected peptide derivatives. The relative strength and the selectivity of the S component in the binary mobile phase were examined on silica gel columns.

The solutes were di- and tri-peptide esters, the N-terminals of which were protected by benzyloxycarbonyl (Z) and tert-butoxycarbonyl (Boc) groups. These compounds were obtained in our laboratory as the synthetic intermediates for the preparation of Angiotensin I. The structures of the solutes are shown in Figure 1.

Correlation between Retention and Binary Solvent Composition

A linear relation between the logarithms of the capacity ratio and the mole fraction of the S component in binary solvents has been observed in silica gel LSC (2-10). The correlation can be expressed as follows:

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

where c and n are constants, k' is the capacity ratio and X_s is the mole fraction of S in the binary system. The retention data obtained for B- and AB-diluent systems and the mole fraction of the S components were plotted using logarithmic scales. The experimental results are illustrated in Figure 2. The two constants were calculated by the least squares procedure and are listed in Table I.

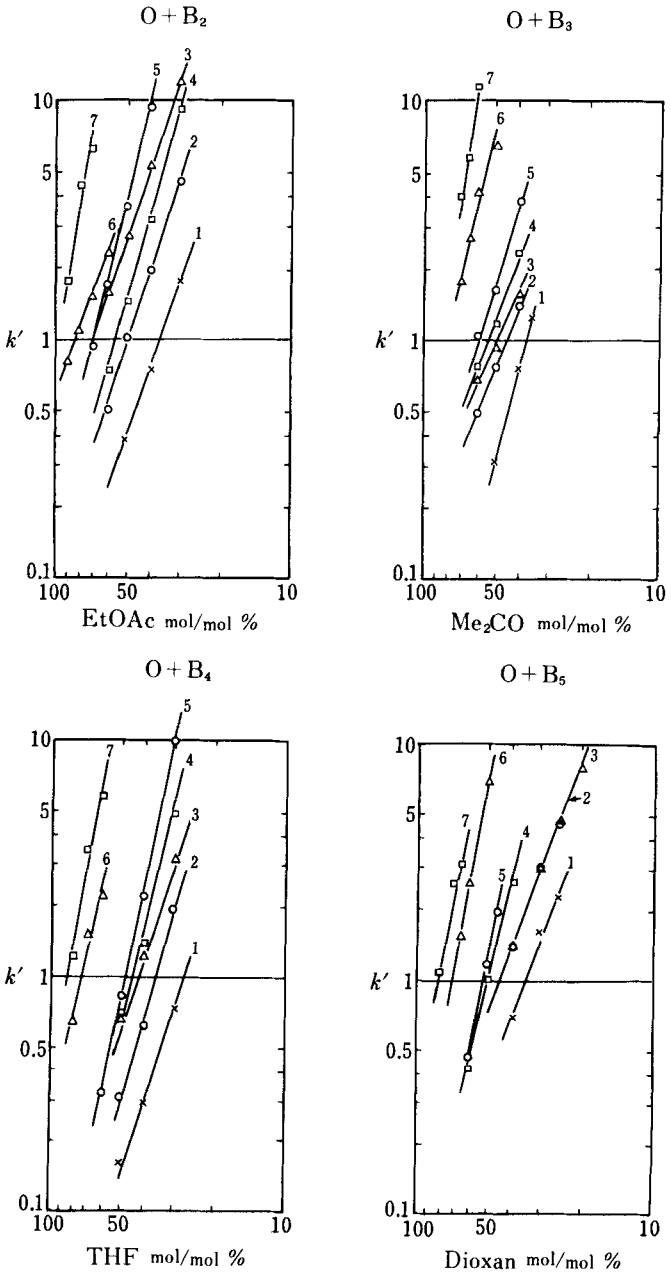
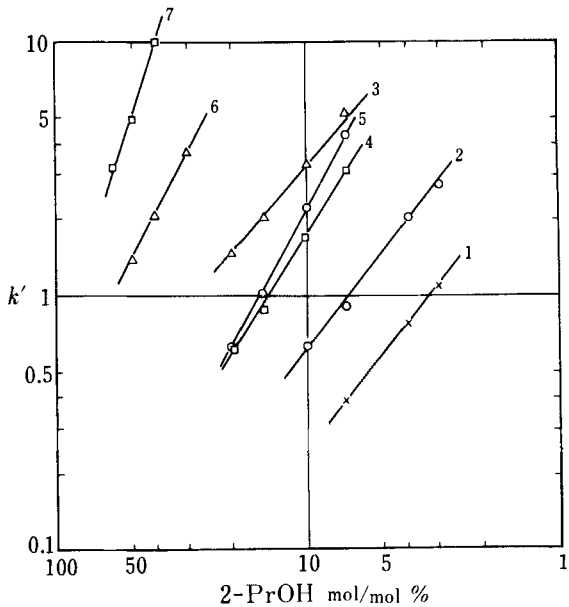


FIGURE 2. Logarithm of capacity ratios on silica gel as a function of the logarithm of the stronger solvent concentration in n-hexane, benzene, dichloromethane, and chloroform-stronger solvent binary systems. Samples as in Figure 1.

O + AB



P + B_4

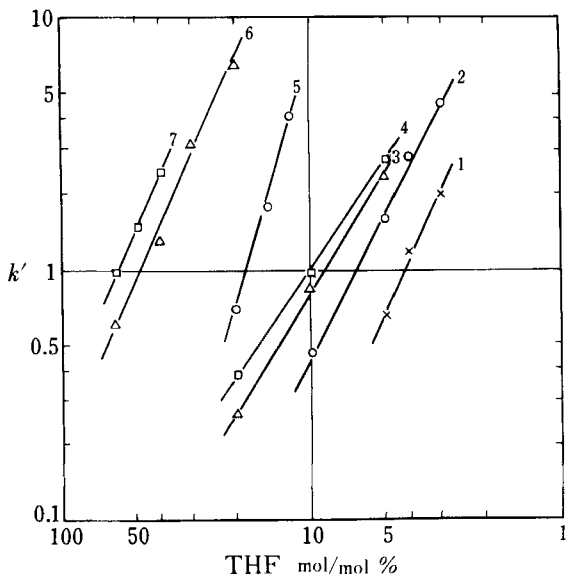
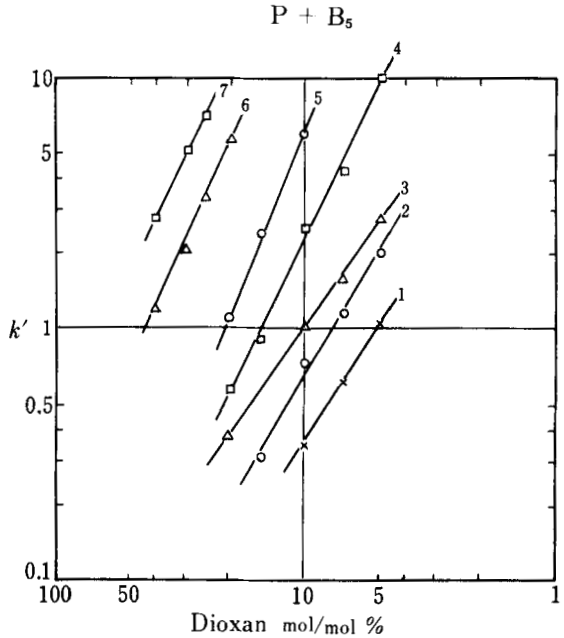


Figure 2 (Continued).



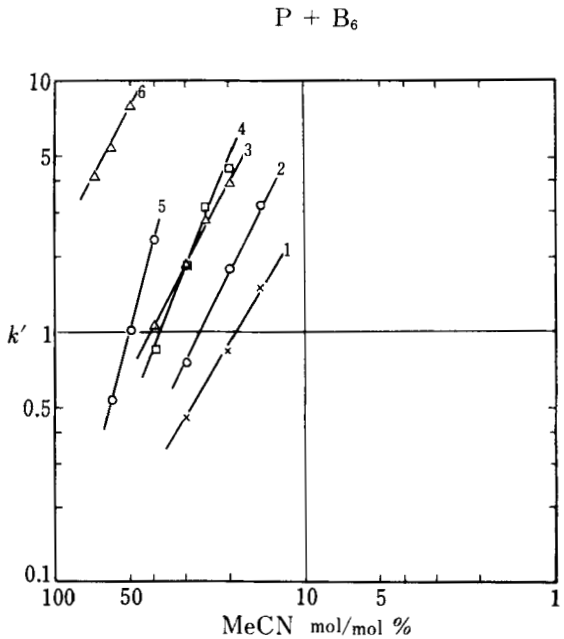
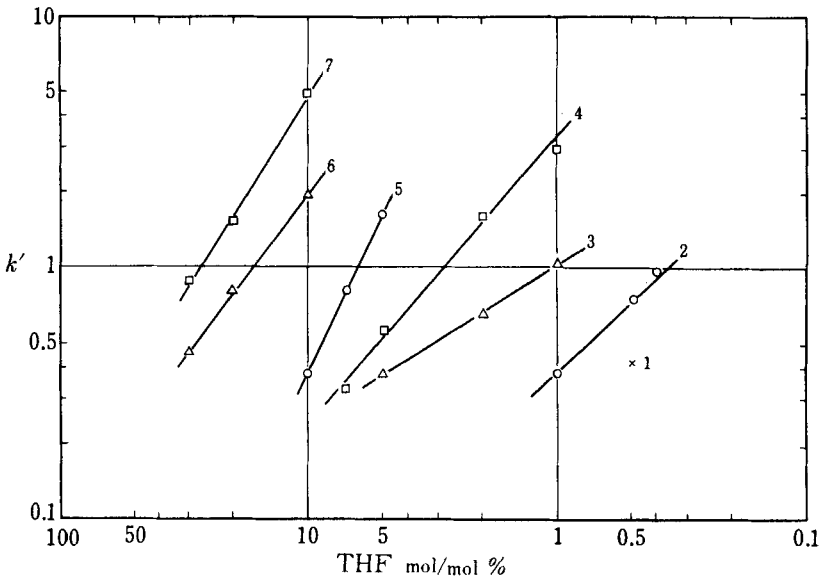
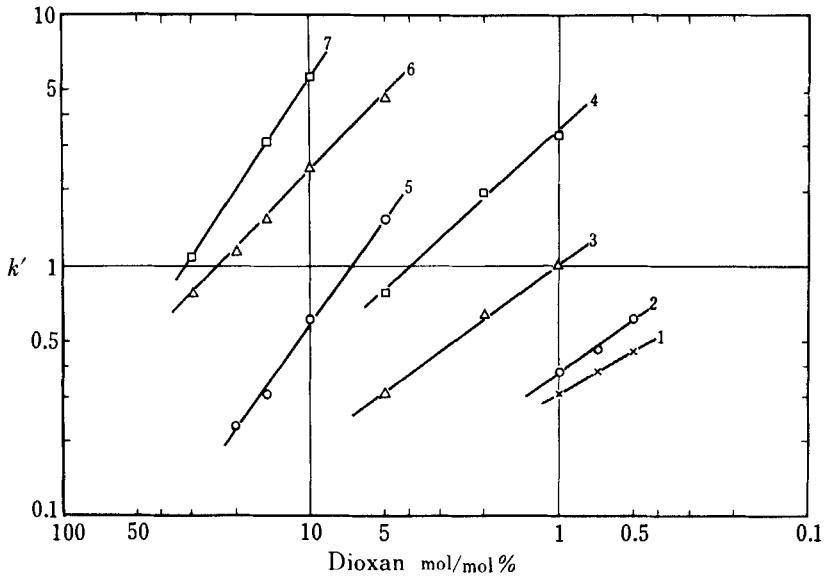


Figure 2 (Continued).

$N_1 + B_4$



$N_1 + B_5$



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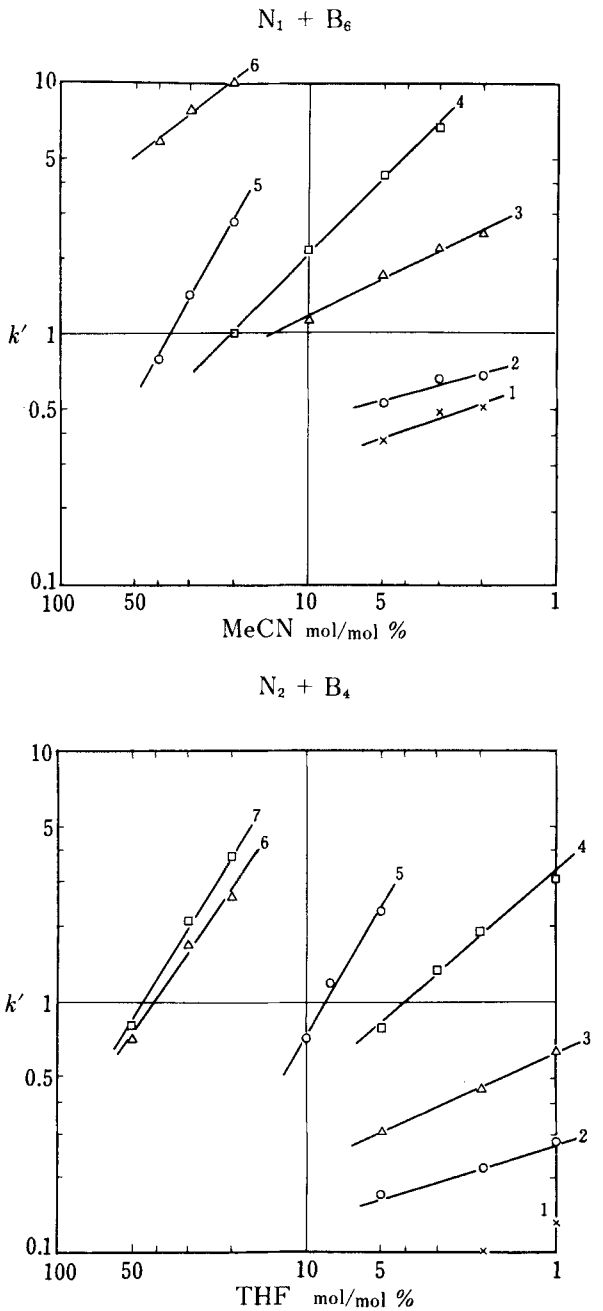
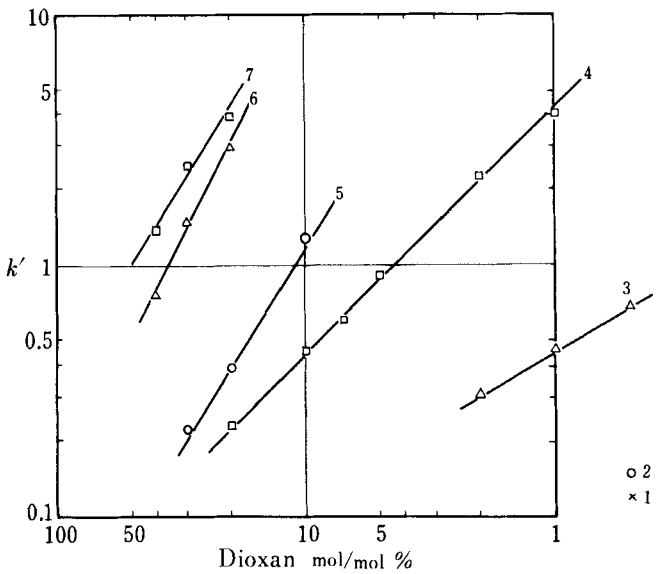


Figure 2 (Continued).

N_2 + B_5



N_2 + B_6

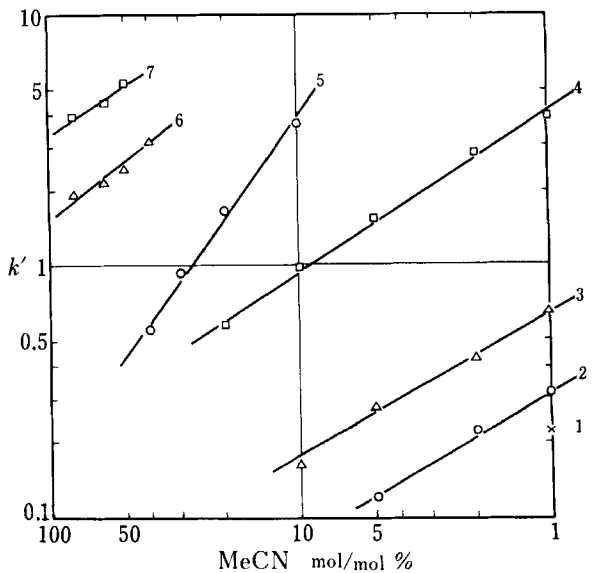


Figure 2 (Continued).

TABLE I

Constants for the Linear Relationship between Retention and Solvent Composition

Sample[c]	O + B_2[a]		O + B_3		O + B_4		O + B_5	
	c[b]	n[b]	c	n	c	n	c	n
1	4.56	2.93	6.42	4.08	4.34	2.99	4.11	2.68
2	5.35	3.17	4.15	2.51	5.53	3.54	4.46	2.71
3	5.39	2.91	3.39	2.01	5.05	3.09	4.37	2.65
4	6.40	3.68	4.86	2.81	6.44	3.91	7.22	4.26
5	7.61	4.15	5.61	3.21	8.19	4.89	8.60	5.03
6	4.92	2.56	7.51	3.90	8.03	4.30	10.39	5.62
7	11.28	5.64	12.88	6.67	10.40	5.39	10.19	5.33

	O + AB		P + B_4		P + B_5		P + B_6	
	c	n	c	n	c	n	c	n
1	0.62	1.23	1.33	2.14	1.12	1.57	2.12	1.68
2	1.04	1.26	1.55	1.92	1.47	1.66	2.80	1.98
3	1.78	1.26	1.48	1.57	1.42	1.41	3.10	1.92
4	1.79	1.55	1.43	1.43	2.42	2.05	3.87	2.45
5	2.22	1.88	4.30	3.43	3.40	2.58	6.09	3.57
6	3.41	1.93	3.68	2.19	3.73	2.29	4.17	1.93
7	5.28	2.69	3.88	2.18	3.66	2.00		

	N_1 + B_4		N_1 + B_5		N_1 + B_6	
	c	n	c	n	c	n
1			-0.51	0.57	-0.16	0.35
2	-0.42	0.94	-0.43	0.70	-0.07	0.26
3	0.02	0.63	0.03	0.74	0.56	0.50
4	0.54	1.15	0.54	0.91	1.32	1.03
5	1.67	2.09	1.17	1.40	2.82	1.82
6	1.58	1.29	1.41	1.02	2.18	0.88
7	2.28	1.60	2.25	1.49		

	N_2 + B_4		N_2 + B_5		N_2 + B_6	
	c	n	c	n	c	n
1	-0.89	0.38				
2	-0.56	0.31			-0.50	0.59
3	-0.20	0.45	-0.34	0.58	-0.18	0.56
4	0.52	0.85	0.63	0.97	0.64	0.62
5	1.52	1.65	1.63	1.56	1.98	1.38
6	2.31	1.44	2.99	1.93	1.70	0.76
7	2.64	1.60	2.70	1.60	1.81	0.64

a Solvents: O=n-hexane; P=benzene; N_1=dichloromethane; N_2=chloroform; B_2=ethyl acetate; B_3=acetone; B_4=tetrahydrofuran; B_5=dioxan; B_6=acetonitrile; AB=2-propanol.

b c, n: constants for Equation (1).

c Samples as in Figure 1.

Parallel retention behaviour was observed, as shown in Figure 2, among three groups of solutes. The groups consisted of three Z-protected dipeptide esters containing phenylalanine as the C-terminal element (1, 2, 3), two Z-protected di- and tri-peptide ethyl esters containing tyrosyl-isoleucine as the C-terminal (4,7) and two of Boc-N-benzyl-hystidinyl di- and tri-peptide esters (5,6). The order of retention strengths is: $1 < 2 < 3$; $4 < 7$; $5 < 6$. The results were interpreted partly in terms of the degree of oligomerization (di-< tri-peptide, i.e., $4 < 7$) and the bulkiness of the alkyl group in the C-terminal esters (tert-butyl < ethyl ester, i.e., $1 < 2$). Steepness of the linear relation for the tripeptides (5,7) is greater than that for the di-peptides (1, 2, 3, 4). Whereas the retention order: $1 < 2 < 3 < 4 < 5 < 6 < 7$ was generally observed in binary systems containing B_4, B_5 and B_6, the sequence of compounds 3, 4 and 5 was exceptionally disordered in O + B_2 and O + AB systems.

The value of the two constants in formula (1) for a particular solvent decreased as the polarity of the diluents and the stronger eluents increased. The order of the eluting strength for the diluents is: $O < P < N_1 < N_2$. The strengths of B_3, B_4 and B_5 were found to be approximately equal in binary systems containing n-hexane (O) as the diluent. Binary systems containing B_6 were weaker than B_4 and B_5 systems. It was concluded that the order of eluting strengths of S components was: $B_4 \geq B_5 \geq B_3 > B_6 > B_2$.

Design of Equi-eluotropic Binary Solvent Systems

To quantitatively determine the relative strengths of all basic and basic/acidic eluents in binary systems, data given in a previous paper (2-4) were incorporated and compared with the results obtained in the present study.

A general procedure for finding concentrations of the weaker component in binary mobile phases with an approximately equal elution strengths was established in a previous article (4,5), and the design of an equi-eluotropic binary solvent system based on the linear relationship expressed by equation (1) became feasible.

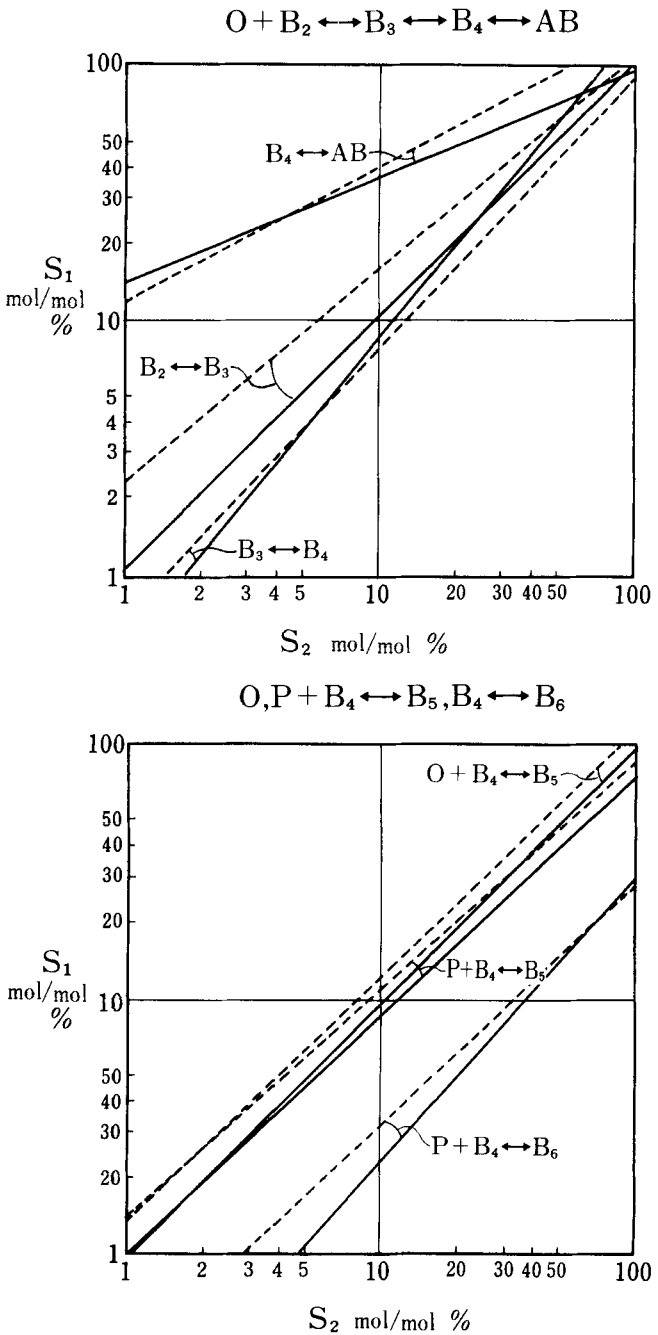


FIGURE 3. Correlation between the concentrations of the stronger components for a pair of equi-eluotropic binary solvent systems with two diluents. Interchange for S components, S_1 to S_2.

This expression is:

$$\log X_{s(1)} = \underbrace{\frac{c_1 - c_2}{n_1}}_{(I)} + \underbrace{\frac{n_2}{n_1} \log X_{s(2)}}_{(II)} \qquad (2)$$

where $X_{s(1)}$ and $X_{s(2)}$, and c_1, c_2, n_1 and n_2 are the molar ratios of S and the two constants in equation (1) for a pair of equi-eluotropic solvent systems 1 and 2, respectively.

Constants for terms I and II were thus calculated for each solute compound. It was shown that the two constants varied rather widely for individual samples, particularly in the case of O + B_2 versus O + B_3 equi-eluotropic binary systems. The amount of scattering observed in the data for the protected peptides is always larger than that in the corresponding data given by steroids (4). Such a larger deviation in the two constants indicates that the solute-solvent interaction for protected peptides must be markedly specific, possibly because the solutes contain a variety of functional groups which are involved in associations with the solvent molecules in the chromatographic system.

In spite of such a large deviation, the average values of the two constants were found to vary no more than those obtained for steroids. Some of the examples comparing two S components are illustrated in Figure 3. Relative strengths obtained for protected peptides are shown by solid lines and those obtained for steroids (4, 5) are shown by dotted lines. In the figure, the average values of the constant terms I and II in equation (2) given by all of the samples as related to the weak components O and P are plotted. The correlation between the two concentrations of stronger components for a pair of equi-eluotropic solvent systems containing O or P as diluent, $X_{s(1)}$, $X_{s(2)}$ is directly indicated.

The experimental results suggest that the binary solvent systems described in this report are suitable for separation of a variety of protected oligo-peptides. It becomes obvious that the procedure for design of binary solvent systems employing the linear relations of the logarithmic values between capacity ratio versus

the concentration of the stronger component and the concentration of a pair of two stronger components in an equi-eluotropic solvents broadens the spectrum of the mobile phases available in LSC. This principle can be useful for the systematic preparation of optimal chromatographic phases having preferable eluting strength and selectivity.

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