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Efficiency in chiral high-performance ligand-exchange chromatography

Influence of the complexation process, flow-rate and capacity factor

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ABSTRACT

Chromatographic separations of enantiomers by ligand-exchange mechanisms are performed either by using chiral stationary phases (CSPs) or chiral mobile phase additives (CMAs). The two modes differ significantly with respect to their efficiency. It is shown that the process of chelate formation and dissociation at the CSP is the main source of enhanced plate height values when employing CSPs. When using highly soluble CMAs as selector ligands, the rate-determining ligand-exchange process takes place mainly in the mobile phase and therefore does not contribute much to the plate heights. These fundamental differences in the kinetics of the two modes of ligand-exchange chromatography are reflected in different dependences of the plate height data on the flow-rates. It is shown that for CSPs the plate height depends on the structure and configuration of the analyte and the composition of the mobile phase. Within a series of homologous amino acids a pronounced positive correlation is found between the kinetic plate height contribution and the capacity factor. However, this positive correlation is not a general dependence: on changing the capacity factor by changing the eluent composition, a negative correlation is found.

INTRODUCTION

Ligand-exchange chromatography (LEC) is a powerful tool for the separation of chiral analytes that form chelate complexes with heavy metals [1,2]. Many applications have illustrated the high enantioselectivity and the great separation power inherent in this method [1–28]. The probable structures of various types of complexes and the correlation of these structures with the observed enantioselectivities have been the topics of extensive investigations, and the results have been reviewed several times [1,2].

Chiral selectors in LEC are either immobilized at the surface of the packing or are components of the mobile phase. The immobilization can be done by binding the selectors chemically to the support material or by dynamically generating an adsorption layer on the support surface. In the latter instance a long hydrophobic

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group acts like an anchor in the alkyl chains of alkylsilica packings. In both instances, chiral stationary phases (CSPs) are obtained. A chiral mobile phase is produced by adding a highly soluble chiral additive to the mobile phase. Whether a chiral mobile phase additive (CMA) is adsorbed or remains predominantly in the mobile phase depends strongly on its activity coefficient in the eluent mixture.

This paper reports the first part of a broader investigation dealing with peak dispersion in high-performance ligand-exchange chromatography (HPLEC). It focuses on differences in peak dispersion found when applying different modes of ligand exchange, either with CSPs or with CMAs. It discusses the effects of flow-rate, the capacity factor and the structural features of the analytes.

THEORETICAL

Plate height equations

The theoretical background for the discussion of plate height data within this paper is provided by the plate height theories for packed columns as given by Huber [29,30] and Giddings [31] (see also a review [32]). These theoretical treatments belong to the most rigorous types usually applied in chromatography.

According to Huber [29,30], we assume that the total reduced plate height, h, is the sum of four different contributions, h_d, h_c, h_f and h_b, originating from different dispersion proceses as follows:

$$h_d = \varphi_d \cdot \frac{D_m}{u d_p} \tag{1a}$$

$$h_c = \frac{\varphi_c'}{1 + \varphi_c'' \left(\frac{D_m}{u d_p}\right)^{\frac{1}{2}}} \tag{1b}$$

$$h_f = \varphi_f \left(\frac{u d_p}{D_m}\right)^{\frac{1}{2}} \left(\frac{k''}{1 + k''}\right)^2 \tag{1c}$$

$$h_b = h_{b,mt} = \varphi_b \cdot \frac{u d_p}{D_m} \cdot \frac{k''}{(1 + k'')^2} \tag{1d}$$

where the subscripts d, c, f and b indicate dispersion due to axial diffusion, convection (including eddy dispersion), resistance to mass exchange in the mobile zone and resistance to mass exchange in the fixed bed, respectively, mt indicates the mass transfer contribution in the stagnant zone, d_p is the mean particle size, u the flow velocity, k' the capacity factor and D_m the diffusion coefficient in the mobile phase; k'' denotes the zone capacity factor, which is related to the phase capacity factor, k', by $k'' = (V_m/V_f)(k' + 1) - 1$, V_m being the mobile phase volume and V_f the interparticle volume; the parameters φ_d, φ_c, φ_f and φ_b are geometric factors depending on the packing material and packing geometry.

Since eqn. 1d accounts mainly for the resistance to mass exchange by the stagnant mobile phase, we have added a particular kinetic term, $h_{b,kin}$, for systems

involving slow adsorption–desorption processes. Following Giddings [31], we assume that $h_{b,kin}$ is given by

$$h_{b,kin} = \varphi_{kin} \frac{u}{d_p k_d} \frac{k'}{(1 + k')^2} \quad (2)$$

where k_d is the rate constant for the desorption of the solute from the surface and φ_{kin} is a geometric factor.

Equilibria involved in ligand-exchange chromatography

We assume that various equilibrium steps are involved in ligand-exchange chromatography, as shown in Fig. 1. Some of these steps are complexation reactions, others adsorption steps without complexation. The rate constants of these two types of equilibrium reactions are expected to differ considerably[a]. Fig. 1 contains those equilibria which we assume to be primarily important for the two different modes of LEC, using either CSPs or highly soluble CMAs. Other equilibria, not specified in Fig. 1 by a number, are assumed either to be of minor importance or not to influence significantly the peak dispersion. Buffer ions, counter ions and solvent molecules involved in the complexes are omitted from Fig. 1 for the sake of clarity. Nevertheless, they may be of great importance as ligand binding is always associated with the dissociation of another ligand. The following equilibria are of most importance:

Equilibria in ligand exchange at CSPs:

(1) Loading of the CSP with metal ions. Usually this loading is already achieved on equilibrating the CSP with a copper-containing eluent before starting chromatography.

(2) Ligand-exchange step of the analyte on the adsorbed metal (we assume this to be the essential rate-determining step).

(3) Adsorption–desorption of the pure analyte without any complexation, *e.g.*, by hydrophobic adsorption (we assume this process to be decisively more rapid than the ligand-exchange process).

(4) Adsorption of the pure analyte onto the stationary phase.

(5) Adsorption of the analyte–metal–analyte complex onto the stationary phase.

Ligand exchange employing highly soluble CMAs:

(1) Formation of the diastereomeric analyte–metal–selector complex in the mobile phase.

(2) Formation of the analyte–metal–analyte complex in the mobile phase.

If these two processes take place almost completely in the mobile phase, the ligand-exchange kinetics do not have much influence on the plate height.

(3) Adsorption of the diastereomeric analyte–metal–selector complex on the stationary phase.

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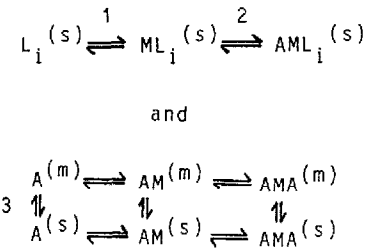
[a] Free energies of binding for hydrophobic adsorption are about 20 kJ/mol [33], whereas those for chelate formation with amino acids are about 80–100 kJ/mol [34,35]. Activation energies for the desorption from hydrophobic binding sites are estimated to be *ca.* 35 kJ/mol [36]. This value is less than the complex binding energy itself and thus in any case much less than the activation energy of complex dissociation. Note that activation free energies for ligand-exchange reactions at surface immobilized ligands might be higher than those for bulk solutions.

(a) CSP:



(b) CMA:

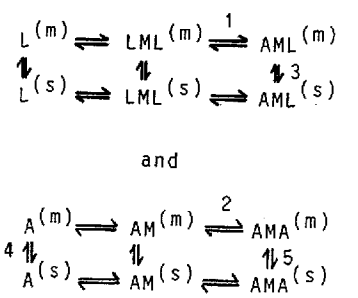


Fig. 1. Schematic representation of complex building and adsorption equilibria involved in LEC: (a) LEC with CSPs; (b) LEC with highly soluble CMAs and metal ion additives. The most important equilibria referred to in the text are indicated by numbers. A = Analyte; M = metal ion; L = chiral selector ligand. Subscript i indicates immobilization; m and s denote species in mobile and stationary phase, respectively.

The three adsorption steps (3)–(5) in Fig. 1b do not involve chelate formation or dissociation steps. They are assumed to proceed with the usual rates of reversed-phase adsorption.

This model implies significant differences in plate heights for the two different ligand-exchange modes (using either CSPs or CMAs). The experimental data on the kinetics of the systems are interpreted in the light of the model described.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a high-performance liquid chromatographic pump (Model L-6200 intelligent pump; Merck–Hitachi, Tokyo, Japan), a syringe–valve injector (Model 7161; Rheodyne, Cotati, CA, U.S.A.) equipped with a 20-μl loop (in specified instances 5 μl), a column oven (Model 655A-52; Merck–Hitachi), a UV detector (Model L-4000; Merck–Hitachi) or a spectrofluorimetric detector (Model F-1000; Merck–Hitachi) equipped with a 2-μl detection cell and an integrator (Model D-2000 chromato-integrator; Merck–Hitachi).

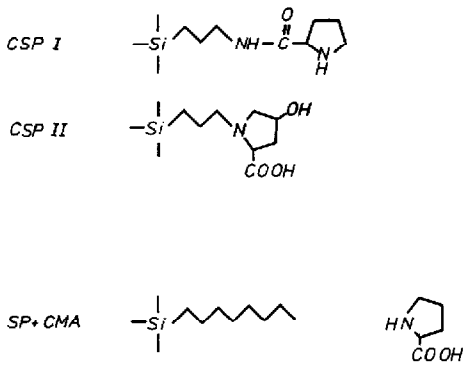


Fig. 2. Structures of the chiral stationary phases CSP I and II and the chiral mobile phase additive.

Columns

Two different CSPs (Fig. 2) were tested:

(i) CSP I: L-proline amide according to Lindner [5]; spacer, propylsilyl, 7-μm particles, material obtained from Loba Chemie (Austria); column dimensions, 125 mm $\times$ 4 mm I.D.

(ii) CSP II: L-hydroxyproline according to Unger and co-workers [12,13]; spacer, propylsilyl, 5-μm particles; column dimensions, 125 mm $\times$ 4 mm I.D.; this column was obtained prepacked from Macherey, Nagel & Co. (Düren, Germany).

Three different columns packed with CSP I were tested.

Non-chiral alkylsilica phases RP-2 and RP-8 (LiChrosorb; Merck) were used for the measurements with CMAs; column dimensions, 125 mm $\times$ 4 mm I.D.

Mobile phases

The organic eluent components methanol, ethanol and acetonitrile were of LiChrosolv grade and tetrahydrofuran (THF) of analytical-reagent-grade from Merck. Water was distilled twice from a quartz apparatus and additionally purified by passage through an RP-8 column before eluent preparation. The eluent mixtures were filtered and degassed by ultrasonic treatment.

The buffer salts, ammonium and sodium acetate and sodium hydrogensulphate, and also copper acetate and copper sulphate were obtained from Merck. The pH of the buffer solutions was adjusted by adding acetic acid or dilute sulphuric acid, and dilute ammonia or sodium hydroxide solution.

Analytes

Mono-, bi- and tridentate ligands of the types benzoic acid, D,L-amino acids and N-dansylated D,L-amino acids were investigated.

Chromatographic conditions

Flow-rates of 1 ml/min and a temperature of 30°C were applied unless indicated otherwise. Careful thermostating was necessary in order to keep the amount of copper adsorbed constant. Generally, UV detection was performed at 254 nm. Fluorescence detection (excitation at 340 nm and emission measurement at 480 nm) was used for

systems with CMAs only. An injection volume of 20 μl was used for experiments employing CSPs and 5 μl for the experiments with CMAs. Void volumes of the columns were determined from the system peaks in copper-free systems. Their values were about 1.20 ml for the given column dimensions.

Significance of data

Plate height values were calculated from peak widths determined at 0.607 of the peak heights and, for control purposes, from the distance between the inflection tangents at the base line [37].

The precision [relative standard deviation (R.S.D.)] of the capacity factor determination was about 3%. The precison (R.S.D.) of plate height determinations was about 10% in LEC systems with bonded selectors and about 7% in reversed-phase systems with CMAs.

The precision and accuracy of plate height determinations in LEC systems with bonded selectors are generally inferior to those in common reversed-phase systems. This is caused by three main factors: (i) with many LEC stationary phases, peak tailing occurs down to low concentrations of analytes, and often one has to balance between systematic errors caused by tailing peaks and statistical errors caused by the scatter of the baseline; (ii) mobile phases in LEC usually contain several components which give rise to several system peaks not all of which are detectable, and system peaks may affect the peak width of the analytes [38], even if the system peaks are undetected; (iii) when detection is performed at a wavelength where mobile phase components absorb, UV-monitored system peaks sometimes interfere with the analyte peaks or disturb the baseline (for this reason some entries in the data tables are blank).

RESULTS AND DISCUSSION

Plate heights in LEC as a function of flow-rate

The dependence of the plate height on the flow-rate is shown for ligand-exchange chromatography with chemically bonded selectors (CSPs) in Fig. 3 and for a system employing a highly soluble chiral selector as CMA in Fig. 4. The curves show significant differences between these two LEC modes.

Plate height curve for the CSP mode. With bonded selector ligands (CSP), the plate heights of most analytes decrease strongly and fairly linearly with the flow-rate, w. The plate height minimum is located at very low values of w. Flow-rate is thus one of the most important and practical parameters for improving the resolution in ligand-exchange chromatography with CSPs.

The strong flow-rate dependence of h supports the assumption that enhanced peak dispersion in LEC with CSPs results primarily from kinetic sources and does not originate from energetic inhomogeneity of adsorption sites and the resulting non-linear adsorption isotherm [39].

The data in Fig. 3 indicate the following pattern:

The plate height is most strongly determined by the dentation number: the monodentate ligand benzoic acid has lower plate height values than the bidentate ligands alanine, valine and leucine. Histidine often acts as a tridentate ligand [6]. The effect of dentation is observed to be predominant over the influence of capacity factors (*cf.*, benzoic acid, D-alanine, D-histidine).

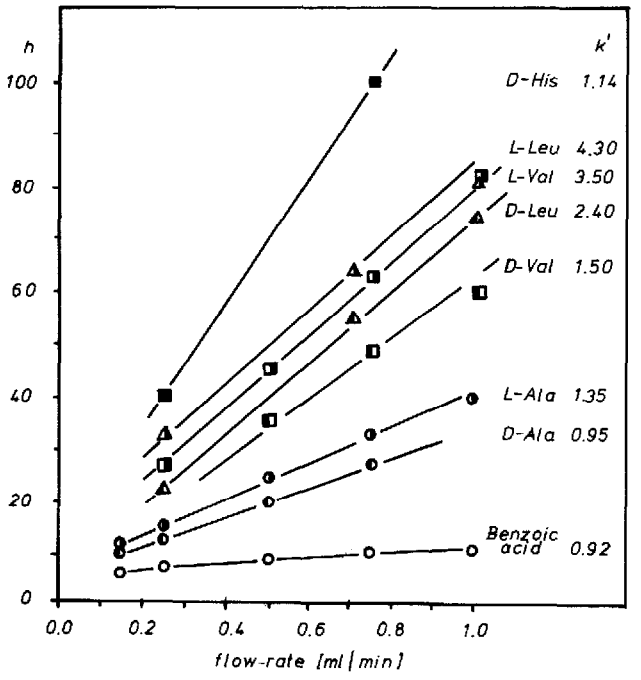


Fig. 3. Influence of flow-rate on the plate height in systems with bonded selectors. Chromatographic conditions: stationary phase, CSP II; mobile phase, aqueous buffer solution [$4 \cdot 10^{-4}$ *M* copper(II) acetate–$5 \cdot 10^{-2}$ *M* ammonium acetate, pH 5.5]; temperature, 30°C.

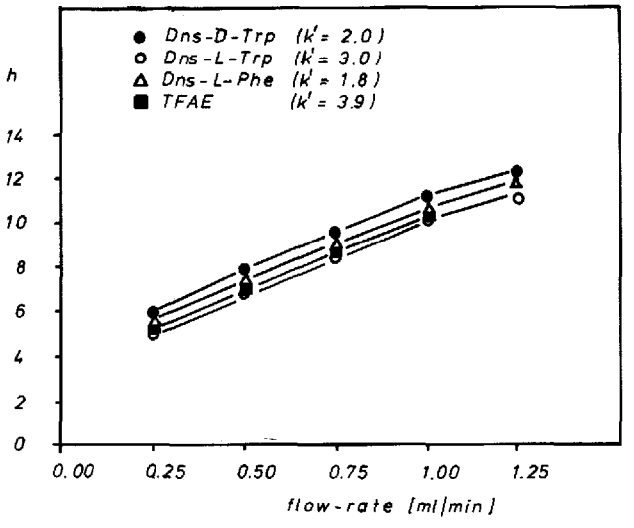


Fig. 4. Influence of flow-rate on the plate height in systems with chiral mobile phase additives. Chromatographic conditions: RP-2 column; mobile phase, aqueous buffer solution [$5 \cdot 10^{-3}$ *M* L-proline–$2.5 \cdot 10^{-3}$ *M* copper(II) sulphate–$1 \cdot 10^{-3}$ *M* ammonium acetate, pH 7]–acetonitrile (75:25, v/v); temperature, 30°C; mobile phase for TFAE, aqueous buffer (10^{-3} *M* ammonium acetate, pH 7)–acetonitrile (60:40, v/v); injection volume, 5 μl.

For the series of bidentate amino acids, the plate heights seem to increase with increasing capacity factors. More detailed investigations concerning the influence of k', however, revealed that the influence of k' is not simple. This point is discussed below.

In all instances investigated, the enantiomer eluted later shows a larger value of h.

Systems employing chiral ligands as CMA. In ligand-exchange systems working with highly soluble chiral mobile phase additives, the dependences of the plate height on the flow-rate are very similar to those usually observed in simple reversed-phase chromatography (Fig. 4). In our experiments, trifluoranthrylethanol (TFAE) was selected as a reference compound in copper-free systems to account for the simple reversed-phase adsorption mode (dansylamino acids have very small capacity factors under these conditions). In comparison with Fig. 3, the slope of the h *vs.* w plot is much smaller, the dependence of h on the capacity factor is marginal[a] and one can only detect a very small, probably insignificant, influence of the optical configuration on h.

The data in Figs. 3 and 4 are the main source for our conclusion that in the CMA mode employing highly soluble chiral additives, and under the chosen conditions, the ligand-exchange equilibrium (equilibrium 1 in Fig. 1b) is not rate determining. It is likely that complexation takes place in both phases, but mainly in the mobile phase, and that the diastereomeric complexes are adsorbed and desorbed from the alkylsilica surface (equilibrium 3 in Fig. 1b) with the rapid kinetics typical of reversed-phase chromatography. Under these conditions slow ligand-exchange kinetics should not have much effect on h, in contrast to the case where the ligand is bound at the surface. Moreover, it is likely that ligand exchange is even more rapid if performed in the mobile phase, owing to fewer steric constraints.

Influence of capacity factor on plate height in the CSP mode

Capacity factors influence the plate height in the CSP mode by a number of mechanisms, which are reflected both in theory (eqns. 1 and 2) and in experimental data.

Experimental data. Within the series of the homologous amino acids investigated and under constant phase system conditions, the plate heights increase from alanine, serine, methionine to valine, leucine and phenylalanine parallel to the increase in the k' values (Table I and the broken lines in the Figs. 5 and 6).

When changing the capacity factors by varying the content of organic modifier in the mobile phase, the h values for a given analyte decrease with increasing k' (full lines in Fig. 5). However, within the series of amino acids, the previously mentioned correlation is maintained (broken lines).

When changing the capacity factors by varying the pH of the eluent, the h values either increase (CSP I) or decrease (CSP II) with increasing k' (Fig. 6 and Table II). In all instances, however, the increase within the series of homologous amino acids is maintained (broken line).

Within the series of free amino acids investigated, the h *vs.* k' correlations are found to be similar in type for the two differing CSPs (Figs. 5 and 6). Within the series

[a] The slight decrease in h observed with increasing capacity factors might originate at least partly from extra-column contributions.

TABLE I

CAPACITY FACTORS, k', AND REDUCED PLATE HEIGHTS, h, AS A FUNCTION OF THE ANALYTE STRUCTURE AND THE VOLUME FRACTION OF ETHANOL IN THE MOBILE PHASE

Stationary phase, CSP II; mobile phase, aqueous buffer [0.05 M sodium acetate–$5 \cdot 10^{-4}$ M copper(II) acetate, pH 5.5]–ethanol as indicated; temperature, 30°C; flow-rate, 1 ml/min.

Amino acid		Ethanol (%, v/v)							
		0		20		40		70	
		k'	h	k'	h	k'	h	k'	h
Non-derivatized									
Benzoic acid		0.70	15	0.38	12				
Ala	I	0.75	31	1.66	28				
	II	0.95	38	2.20	31				
Met	I	1.82	38	3.09	38				
	II	2.52	47	3.79	44				
Val	I	1.36	50	2.39	43				
	II	2.86	58	4.70	47				
Leu	I	1.85	50	2.64	46				
	II	2.98	61	3.94	55				
Phe	I	3.44	53	4.65	50				
	II	4.88	60	5.80	58				
Dansylated									
Dns-Val	I			1.76	32	1.53	42	1.98	46
	II			3.61	41	3.18	52	4.25	61
Dns-Leu	I			1.78	29	1.06	25	1.51	38
	II			2.71	37	1.85	35	2.33	41
Dns-Ser	I			3.12	38	3.72	56	5.50	80
	II			5.82	50	5.65	96		
Dns-Phe	I			4.15	51	4.14	62	4.30	53
	II			8.09	76	10.42	130	12.80	110

of dansylated amino acids these h *vs.* k' correlations are different between CSP I and CSP II (Fig. 7).

For CSP II, the plate heights of dansyl amino acids increase strongly with increasing k' (like free amino acids), but for CSP I, h is nearly independent of k' (unlike free amino acids). This independence is also seen from the data in Table III, where the influence of different types of organic modifiers in the eluent is reported.

This difference between the two CSPs is of interest for investigations concerning differences in the predominant adsorption mechanisms in LEC phases. A more detailed discussion of the influence of organic modifiers and pH on the plate height will be given in a future paper in the broader context of a study on mixed adsorption mechanisms. With regard to the influence of the capacity factor, which is the primary concern here, the experimental finding is that no simple dependence of h on k' can be observed.

Theoretical expectations. From eqns. 1 and 2 we expect an increase in h with increasing capacity factors via the contribution h_f, a decrease in h via the contribution

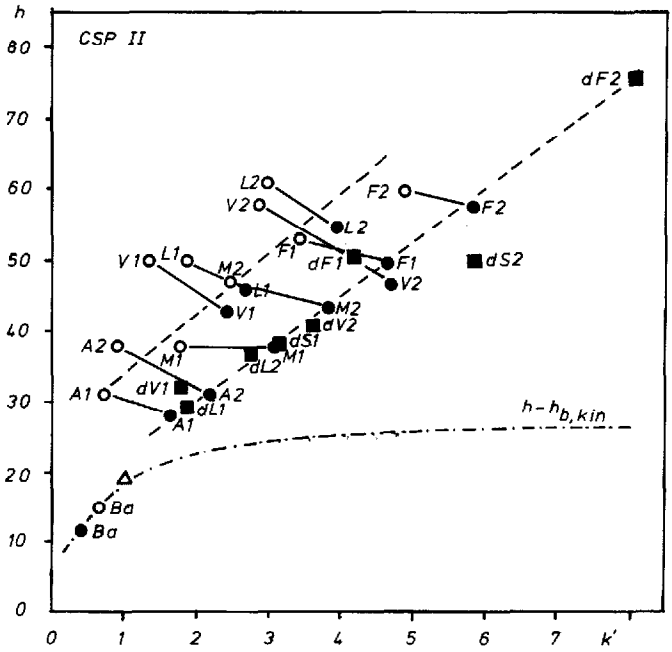


Fig. 5. Plate height *vs.* capacity factor correlation: influence of analyte structure and ethanol concentration in the mobile phase. Stationary phase, CSP II; mobile phase, aqueous buffer solution [$4 \cdot 10^{-4}$ *M* copper(II) acetate–$5 \cdot 10^{-2}$ *M* ammonium acetate, pH 5.5] with various concentrations of ethanol; temperature, 30°C; flow-rate, 1 ml/min. Circles, free amino acids; squares, dansylated amino acids; open symbols, no ethanol present; full symbols, 20% (v/v) ethanol. Analyte symbols: A = alanine; V = valine; L = leucine; S = serine; M = methionine; F = phenylalanine; W = tryptophan; T = threonine; Ba = benzoic acid; A1 = first-eluted enantiomer of alanine, A2 = second-eluted enantiomer, etc.; dA = dansylalanine; dV = dansylvaline, etc. Triangle: data point obtained for Dns-Phe in a phase system without copper ions; the dot-dashed line indicates plate height values without kinetic contributions, $h - h_{b,kin}$, obtained by extrapolation.

$h_{b,mt}$ and, depending on the values of the dissociation rate constant, k_d, and of k', an increase or a decrease via the kinetic contribution $h_{b,kin}$. Typical values for the k'-related factors in these terms in the investigated k' range are given in Table IV. An approximate evaluation of the relative contributions of the mentioned plate height terms gives the following:

For $h_{b,mt}$ we know from previous investigations that this term is small [40–42]. In a reasonable approximation one can therefore neglect this term in this discussion.

h_f might be important. Between $k' = 1$ and 10 this contribution increases by about 60% (*cf.*, Table IV). From data for copper-free systems we are able to calculate approximate h values for the case of insignificant kinetic contributions (in this instance the dependence on k' is mainly due to the h_f term). For CSP I experimental h *vs.* k' data in copper-free systems are available between $k' = 3$ and 14 and are given in Fig. 8. These data are plotted as the $h - h_{b,kin}$ curve at the bottom of Fig. 6b. For CSP II fewer experimental h data in copper-free systems are available. The extrapolated $h - h_{b,kin}$ curve at the bottom of Fig. 5 is obtained by assuming that under the given conditions at

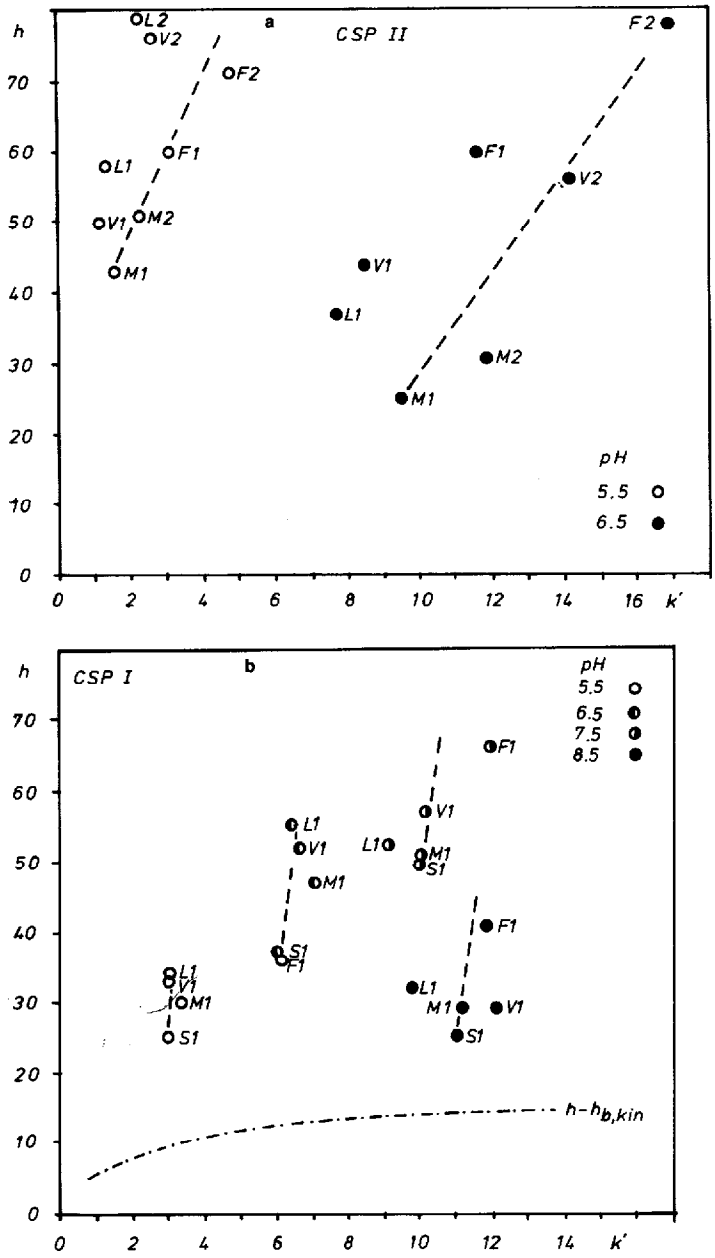


Fig. 6. Plate height *vs.* capacity factor correlation: influence of analyte structure and pH of the mobile phase. (a) Stationary phase, CSP II; mobile phase, aqueous buffer solution [$4 \cdot 10^{-4}$ *M* copper(II) acetate–$5 \cdot 10^{-2}$ *M* ammonium acetate, pH as indicated] with no organic modifier. (b) Stationary phase, CSP I; mobile phase, aqueous buffer solution [$4 \cdot 10^{-4}$ *M* copper(II) acetate–$5 \cdot 10^{-2}$ *M* ammonium acetate, pH as specified]–methanol (70:30, v/v); temperature, 30°C; flow-rate, 1 ml/min. Abbreviations of analytes as in Fig. 5. The dot-dashed line indicates the extrapolated total plate height curve without kinetic contributions, $h - h_{b,kin}$, obtained by use of the data in Fig. 8.

TABLE II

CAPACITY FACTORS, k', AND REDUCED PLATE HEIGHTS, h, AS A FUNCTION OF THE ANALYTE STRUCTURE AND THE pH OF THE ELUENT

(A) Stationary phase, CSP I; mobile phase, aqueous buffer [0.05 M ammonium acetate–$4 \cdot 10^{-4}$ M copper(II) acetate, pH values as indicated]–methanol (70:30, v/v); temperature, 30°C; flow-rate, 1 ml/min. (B) Stationary phase, CSP II; mobile phase, aqueous buffer [0.05 M ammonium acetate–$5 \cdot 10^{-4}$ M copper(II) acetate, pH values as indicated] with no organic modifier; temperature, 30°C; flow-rate 1 ml/min.

Conditions	Analyte		pH							
			5.5		6.5		7.5		8.5	
			k'	h	k'	h	k'	h	k'	h
A	L-Ser		2.98	25	6.04	37	10.01	49	10.97	25
	L-Met		3.42	30	7.09	47	10.04	49	11.16	29
	L-Val		3.10	33	6.72	52	11.98	57	12.18	29
	L-Leu		3.04	34	6.50	55	8.74	52	9.78	32
	L-Phe		6.08	36	10.30	–	11.94	66	11.86	41
	Dns-L-Val		6.47	25	16.18	56	13.71	82	5.00	103
	Dns-D-Val		9.89	28	29.30	54	23.73	79	8.02	–
	Dns-L-Leu		6.56	23	13.30	52	12.42	67	6.15	94
	Dns-D-Leu		10.74	25	28.00	–	23.74	55	10.06	93
	Dns-L-Ser		17.58	29	25.87	–	26.40	66	7.98	–
	Dns-D-Ser		33.06	24	47.80	–	42.46	56	10.54	–
B	DL-Met	I	1.6	43	9.5	25				
		II	2.3	51	11.9	31				
	DL-Val	I	1.2	50	8.5	44				
		II	2.6	76	14.2	56				
	DL-Leu	I	1.3	58	7.7	37				
		II	2.2	79	12.6	–				
	DL-Phe	I	3.1	60	11.6	60				
		II	4.8	71	16.9	78				

$k' = 1$ about half of the total plate height value originates from h_c (which is not dependent on k'), and about half from h_f, which can be extrapolated by means of eqn. 1c. On the basis of this rough approximation, the $h - h_{b,kin}$ curve can serve in this instance as a semi-quantitative guide only.

$h_{b,kin}$ is influenced by k' in two ways (*cf.*, eqn. 2): by the factor $k'/(k' + 1)^2$ and by the dissociation rate constant k_d. The k'-containing factor has its maximum value at $k' = 1$ and decreases to half of this value when k' increases from 1 to 6. In this capacity factor range, this is a negative dependence.

Considering the dissociation rate constant, it should be noted that k_d is determined by the activation free energy of complex dissociation and not by the binding energy of the complex, which determines k'. We expect, however, that under certain conditions (*e.g.*, considering a series of homologous compounds) the activation energy for this "reaction" and the binding energy are correlated to a certain extent. In these instances h will be positively correlated with k' via k_d, and this correlation may

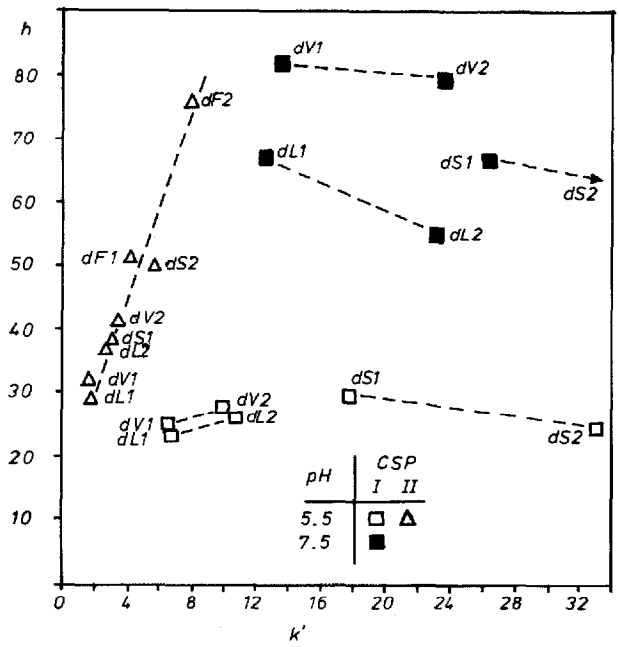


Fig. 7. Plate height *vs.* capacity factor correlation: influence of analyte structure of dansylated amino acids, pH and type of stationary phase. Stationary phases, CSP I and II; mobile phases, aqueous buffer solution [$4 \cdot 10^{-4}$ *M* copper(II) acetate–$5 \cdot 10^{-2}$ *M* ammonium acetate, pH as indicated]–methanol (70:30, v/v) for CSP I and aqueous buffer solution–ethanol (80:20, v/v) for CSP II; temperature, 30°C; flow-rate, 1 ml/min. Abbreviations of analytes as in Fig. 5.

TABLE III

CAPACITY FACTORS, k', AND REDUCED PLATE HEIGHTS, h, AS A FUNCTION OF THE TYPE OF ORGANIC MODIFIER IN THE ELUENT

Stationary phase, CSP I; mobile phase, aqueous buffer [0.05 *M* ammonium acetate–$5 \cdot 10^{-4}$ *M* copper(II) acetate, pH 5.5]–organic modifier (30:70, v/v); temperature, 30°C; flow-rate, 1 ml/min.

Compound		Modifier							
		Acetonitrile		Methanol		Ethanol		THF	
		k'	h	k'	h	k'	h	k'	h
Phe		2.27	13	2.61	14	2.00	15	1.33	14
Ile		2.12	11	3.32	17	2.57	17	1.58	17
Dns-Nval	I	1.50	5.1	8.58	6.6	4.80	6.5		
	II	2.05	5.8	11.74	7.6	6.00	6.0		
Dns-Leu	I	1.40	6.3	7.74	5.6	4.54	6.7	2.37	7.4
	II	1.95	6.2	10.12	6.1	5.54	6.5	2.60	6.1
Dns-Met	I	2.30	5.4	12.0	6.7	6.63	8.1	0.50	6.7

TABLE IV

NUMERICAL VALUES FOR THE CAPACITY FACTOR-RELATED FACTORS IN THE EQNS. 1c, 1d AND 2

k'	k''	$[k''/(k''+1)]^2$ in h_f	$k''/(k''+1)^2$ in $h_{b,mt}$	$k'/(k'+1)^2$ in $h_{b,kin}$
0.1	1.2	0.30	0.24	0.08
0.5	2.0	0.44	0.22	0.22
1.0	3.0	0.56	0.19	0.25
3.0	7.0	0.77	0.11	0.19
5.0	11.0	0.84	0.08	0.14
10.0	21.0	0.91	0.04	0.08
20.0	41.0	0.95	0.02	0.05

mimic a strict and direct dependence of k_d (and thus h) on k', although a strict dependence is neither implied by theory nor found generally in the experiments (*cf.*, Table 7 in ref. 35). This positive correlation can be so significant that it far overcompensates for the negative correlation via the factor $k'(k'+1)^2$.

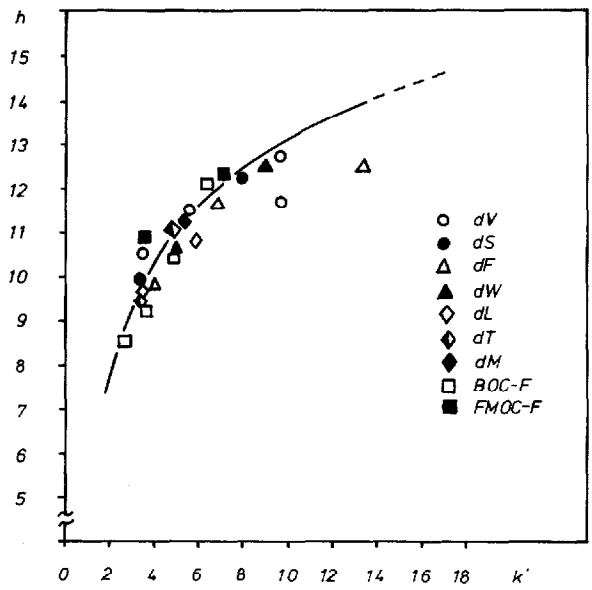


Fig. 8. Plate height *vs.* capacity factor correlation in copper-free systems. Stationary phase, CSP I; mobile phase, aqueous buffer solution ($5 \cdot 10^{-2}$ *M* ammonium acetate, pH 5.5) with various concentrations of acetonitrile; temperature, 30°C; flow-rate, 1 ml/min. Abbreviations of analytes as in Fig. 5. BOC = butyloxycarbonyl-; FMOC = fluorenyloxycarbonyl-.

CONCLUSIONS

From the data reported above, the following conclusions can be drawn:

In systems where ligand-exchange processes are operative at the surface, we found that plate heights for the bi- or tridentate analytes investigated are significantly higher than in systems without ligand-exchange processes (*cf.*, the $h - h_{b,kin}$ lines in Figs. 5 and 6 and the data in Fig. 8 obtained in the absence of metal ions). We attribute this effect to the slow ligand-exchange rates for a ligand-exchange step taking place at the surface of the stationary phase.

LEC systems using CMAs generally have better efficiencies than systems with CSPs. No significant difference in plate heights is observed between ligand-exchange and the usual reversed-phase adsorption chromatography. We conclude that in this mode the dominant mechanism is the adsorption of the diastereomeric complexes, which are formed in the mobile phase, by a simple reversed-phase adsorption mechanism. In this instance, slow ligand-exchange kinetics do not have much effect on h.

The plate height dependences on the flow-rate have different strengths for the two LEC modes. This is in agreement with the given model. As the ligand-exchange specific contribution to h is high with CSPs, the plate height minimum is situated at very low flow velocities. Using LEC with CSPs, there is great potential for improving the resolution by reducing the flow velocity.

When using immobilized selector ligands, the kinetic contribution, $h_{b,kin}$, depends on several parameters:

The most important influence originates from the dentation number of the analyte ligands. This is in accord with the fact that binding energy and the resulting activation energy for dissociation increase strongly with increase in the number of ligand–metal bonds involved.

The influence of the capacity factor on h is not simple. Within a series of amino acids we observe a certain positive correlation between the plate heights and the capacity factors, although there is a fairly reproducible fine structure of systematic deviations from this correlation. Plate heights, however, may also decrease with increasing k', *e.g.*, when varying the eluent composition. However, the positive correlation within the amino acid series mentioned is always maintained, and to a great extent even the "fine structure" of deviations (Figs. 5 and 6). The conservation of these fine structure on variation of the mobile phase precludes its origination from a statistical scatter of data.

From these observations, we conclude that h is not simply determined by the capacity factor itself[a], but rather by a parameter that is related to the length, shape, steric size and bulkiness of the side-chains and to the configuration at the chiral carbon atom of the amino acids, all of which affect the reaction rate constant and thus $h_{b,kin}$ via the free energy of activation. This conclusion is supported by the fact that in general an increase in h with increasing "bulkiness" of the side-chains has been found in all experiments (which measure for "bulkiness" will be most appropriate in this context is not yet clear). It is likely that bulkiness plays an even more important role for the

[a] Note in addition that the experimental h values are not really linearly correlated with the formation constants of the amino acid–copper complexes [35].

chelating reactions at surface-immobilized complexes than for those in a bulk phase.

For the amino acids investigated, the increasing steric size of the side-chains is also correlated with enhanced k' values. We therefore conclude that the observed positive correlation between h and k' within the series of amino acids basically originates from this correlation between binding energy and free energy of activation. It is in good agreement with this explanation that a different dependence of h on k' is detected when k' is increased not by varying the length of the side-chain but by varying the eluent composition.

Similar explanations may hold for the dependence of the reaction rate on the optical configuration. This influences k' and, in the way mentioned before, the energy of the transition state for the complexation reaction.

The different patterns in the h *vs.* k' curves observed for the dansylated amino acids at the two chiral stationary phases probably originate from a mixed mechanism of adsorption on at least one of these phases (CSP I). We have seen from capacity factor data in copper-free systems (triangle symbol in Fig. 5 and data in Fig. 8) that the hydrophobic adsorption of dansylamino acids without complexation is significant with CSP I, unlike the situation with CSP II. We argue that the approximate invariance of h with k', shown for CSP I in Fig. 7, might result from the reduced contributions of complexation for these analytes under the given conditions. This problem is the subject of a broader investigation to be presented in a subsequent paper.

We conclude that the predominant plate height-determining parameters are the dentation number of the analyte and the steric size and the optical configuration of the interacting groups in the chelate complex, all of which influence the dissociation rate constant, k_d. The plate height is not primarily determined by the value of the capacity factor itself. Capacity factors are often correlated with k_d, especially within a homologous series of analytes, but not necessarily.

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