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Enantiomeric separation of dansyl- and dabsylamino acids by ligand-exchange chromatography with (*S*)- and (*R*)-phenylalaninamide-modified silica gel

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ABSTRACT

(*S*)- and (*R*)-phenylalaninamide [(*S*)- and (*R*)-PheA, respectively] were covalently bonded to silica, and the novel chiral stationary phases (CSPs) obtained were used for ligand-exchange chromatographic (LEC) separations with Cu(II) as complexing metal ion. These CSPs perform good separations of dansyl-I (Dns)- and dabsyl-I (Dbs) amino acids. The results [enantioselectivity factor (α), retention factor (k') and elution order] were compared with the data obtained previously using one of the selectors as an additive to the mobile phase. Moreover, these CSPs were successfully employed in the direct resolution of a five-membered ring cyclic sulphonamide (γ-sultam) without any prederivatization procedure. The chiral recognition process was found to be consistent with a ligand-exchange mechanism, the geometry of the mixed diastereomeric complexes being strongly dependent on the structural features of the selector covalently bonded to the phase and on the steric bulk of the Dns- and Dbs-amino acid protecting groups. In order to investigate the nature of the interactions involved in the discrimination process, the influence of temperature on the chromatographic parameters (k', α) was also evaluated.

INTRODUCTION

Ligand-exchange chromatography (LEC) has proved to be very successful for the separation of several enantiomers, especially of amino acids [1–6]. The chiral copper complex was either bonded to the phase [chiral stationary phase (CSP)] [7–10] or added to the eluent [chiral eluent (CE)] [11–15]. In the former instance, the enantiomeric separation is essentially based on the formation of diastereomeric mixed complexes with different thermodynamic stabilities. It is generally accepted that chiral discrimination proceeds via the substitution of one ligand in the coordination sphere of the metal ion [16], although outer-sphere interactions have been proposed [17]. The problem that occurs when the chiral copper complex is added to the eluent is still open: a variety of cases may be envisaged, depending on the nature of the initial copper complex and of the enantiomers.

In a general project aimed at studying the nature of the interactions responsible for chiral discrimination in HPLC, we have previously reported the separation of D,L-dansyl- (Dns) amino acids (dansyl = 5-dimethylamino-1-naphthalene-sulphonyl) on a reverse-phase column (C_{18}) using mobile phases containing copper(II) complexes of L-amino acyl amides [18]. In particular, (*S*)-phenylalaninamide [(*S*)-PheA] gave very good results, and it was also applied to the separation of D,L-amino acids in foods [19].

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On the basis of an accurate study of the initial copper complexes in solution [20] and in the solid state [21] and of the chromatographic parameters (elution order, pH, eluent polarity), the selector (*S*)-PheA copper(II) complex seemed to be a well established case of a "classical ligand exchange" with total displacement of a bidentate ligand and substitution with a bidentate analyte.

In order to obtain further clues on the mechanism of the enantiomeric recognition, we decided to bind (*S*)-PheA covalently to silica gel [22] to compare the results with those obtained previously using the same selector as an additive to the mobile phase [18,23]. In addition, we synthesized a second CSP [(*R*)-PheA-CSP], with reversed chirality, as it is possible to reverse the elution order of the enantiomers by inverting the chirality of the column and to determine traces of an enantiomer in the presence of an excess amount of the other one [24,25]. In particular, in this paper we report the enantiomeric separation of Dns- and dabsyl-(Dbs) amino acids (dabsyl = 4-(dimethylamino)azobenzene-4′-sulphonyl) and the investigation of the discrimination process.

EXPERIMENTAL

Apparatus

Analytical liquid chromatography was performed on a Waters (Milford, MA, USA) chromatograph equipped with an U6K universal injector, two Model M510 solvent-delivery systems and a temperature-control module (TCM). Different detectors were used, including a Model M409 programmable multi-wavelength detector (Waters). Chromatographic data were collected and processed on a Waters Model 840 data and chromatography control station.

^{1}H and ^{13}C NMR spectra were recorded on a Varian (Palo Alto, CA, USA) XL300 spectrometer. Mass spectra were recorded with a VG Tritech (Manchester, UK) TS250 mass spectrometer. IR spectra were recorded as potassium bromide pellets on a Nicolet (Madison, WI, USA) 5DX Fourier transform (FT) IR spectrometer.

Chemicals and reagents

LiChrosorb Si 100 (5 μm particle size) was purchased form Merck (Darmstadt, Germany). HPLC-grade solvents were obtained from Carlo Erba (Milan, Italy), 3-glycidoxypropyltrimethoxysilane (GOPTMS) from Janssen (Beerse, Belgium) and (*S*)-phenylalaninamide [(*S*)-PheA], dabsyl chloride, D-phenylalanine, D-threonine and Dns-amino acids from Sigma (St. Louis, MO, USA). (*R*)-Phenylalaninamide [(*R*)-PheA)] was synthesized according to ref. 26; the Z protector group (Z = benzyloxycarbonyl) was then removed with Pd–C and cyclohexene [27] to give (*R*)-PheA. The product was characterized by FT-IR and ^{1}H NMR spectrometry, elemental analysis, etc.

Racemic and enantiomerically pure amino acids (Ala, Val, Leu, Ser, His, Asp, Met, Glu) were obtained from Fluka (Buchs, Switzerland). Racemic and enantiomerically pure *p*-hydroxyphenylglycine was supplied by Recordati Industria Chimica Farmaceutica (Latina, Italy). Dabsyl (Dbs) derivatives of amino acids were synthesized by standard methods [28]. Isothiazolidine-1,1-dioxide-3-carboxylic acid [29] was kindly provided by Professor G. Lucente (Dipartimento di Studi Farmaceutici, Università "La Sapienza", Rome, Italy). The remaining chemicals were of analytical-reagent grade and used as received.

Preparation of the chiral stationary phase

The chiral stationary phases [(*S*)-PheA-CSP, (*R*)-PheA-CSP] were synthesized from LiChrosorb Si100 (5 μm) silica gel.

Glycidoxypropylsilica gel (GPSG) was obtained as described previously [30,31]. Elemental analysis of the washed (toluene, dichloromethane) and dried (0.1 mbar, 60°C) silica material gave C 5.19%, H 1.11%, equivalent to 618 μmol of epoxide per gram of silica (from carbon).

The chiral ligands were then bonded to GPSG as reported in Fig. 1. Elemental analysis of the washed (dimethylformamide, water, methanol, dichloromethane) and dried (0.1 mbar, 70°C) silica material gave C 7.94%, H 1.23%, N 0.97%, equivalent to 346 μmol of selector (*S*)- or (*R*)-PheA per gram of silica (from nitrogen).

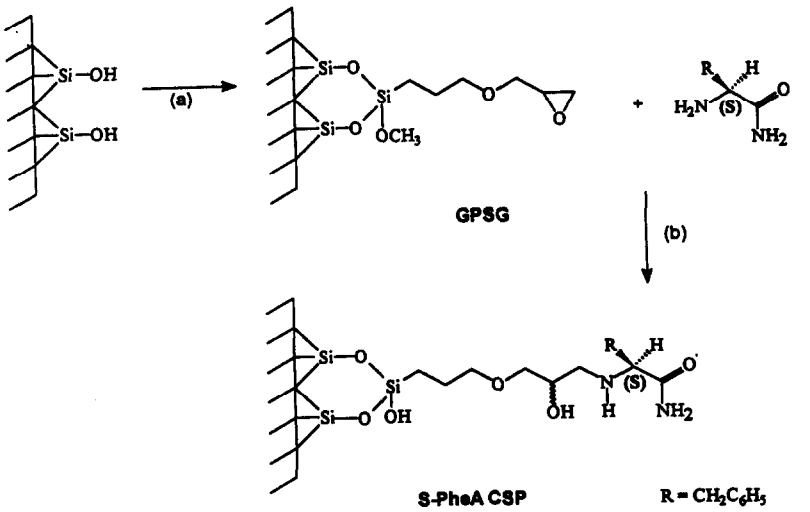

Fig. 1. Synthetic pathway for the preparation of (*S*)-PheA, (*R*)-PheA or the racemic version of CSP. (a) Glycidoxypropyltrimethoxysilane (GOPTMS), toluene, 4 h, reflux; (b) dimethylformamide, 7 days, room temperature. (*R*)-PheA or racemic PheA can also be used.

Column packing and column evaluation

Stainless-steel columns (150 × 4.0 mm I.D.) were packed with (*S*)- or (*R*)-PheA-modified LiChrosorb Si100 (5 μm) using a slurry packing procedure [32].

Grafted silica (*ca.* 1.2 g) was dispersed in chloroform (30–40 ml) and then treated ultrasonically for 5 min. The slurry obtained was packed with a Haskel DSTV-122 pump using *n*-hexane as pressurizing agent (8000 p.s.i., 15 min).

Efficiency test. *n*-Hexane–chloroform (90:10, v/v) at a flow-rate of 1.0 ml/min at room temperature was the eluent used in the evaluation of the kinetic performance of the columns. The test mixture consisted of benzene, nitrobenzene, methyl benzoate, acetophenone and 1,3-dinitrobenzene. Calculation of the number of theoretical plates was done on the last peak (1,3-dinitrobenzene) and was always >45 000–50 000 plates/m.

The column dead volume (V_0) was determined from the elution time of an unretained marker (benzene, using methanol as eluent).

Chromatographic procedures

Before starting the chiral separations, in order to ensure the formation of the proper copper complexes on the stationary phase surface, a solution of copper(II) sulphate (16 m*M*) was allowed to flow through the column for 30 min at a flow rate of 1 ml/min.

The eluents for the separations were mixtures of required percentages of acetonitrile and aqueous solutions of copper acetate and ammonium acetate. The pH was always between 6.0 and 7.5. The mobile phase was then filtered and degassed with helium.

RESULTS AND DISCUSSION

We report the preparation of both (*S*)- and (*R*)-phenylalaninamide-bonded chiral stationary phases [(*S*)-PheA-CSP and (*R*)-PheA-CSP) on siliceous microparticles for use in the enantiomeric separation of amino acid derivatives [dansyl (Dns) and dabsyl (Dbs)] by LEC.

(*S*)- and (*R*)-phenylalaninamide [(*S*)- and (*R*)-PheA] were covalently bonded to silica in a simple manner as reported in Fig. 1. The siliceous matrix was activated with glycidoxypropyltrimethoxysilane, producing the glycidoxypropylsilica gel (GPSG intermediate). In the second step, the modified silica gel (GPSG) was functionalized with the selector phenylalaninamide (*R* or *S* configuration or racemic). Finally, the CSP was repeatedly washed with both organic and aqueous solvents.

As no variation was observed in the physico-chemical properties of the phase, it can therefore be assumed that a column packed with this phase can be eluted with aqueous or organic solvents.

The siliceous matrices were characterized at different steps in the working processes by physico-chemical methods (thermogravimetric analysis, elemental analysis, etc.).

CSP thermogravimetric (TG) analysis, carried out under a nitrogen atmosphere, showed heat resistance; in fact, the TG of these CSPs showed no mass variation up to 200°C.

Resolution of D,L-Dns-amino acids

Several D,L-Dns-amino acids were completely resolved, as shown in Table I. The α values range from 1.40 to 5.54 using CH_3CN/0.1 *M* NH_4OAc–0.25 m*M* $Cu(OAc)_2$ (70:30, v/v) (pH 7.52, 60°C) as the mobile phase.

On (*S*)-PheA-CSP, the elution order was always $k'_D < k'_L$ in contrast to that observed with (*S*)-PheA in the mobile phase [18]; in the latter instance, only for the Dns derivatives of polar amino acids was $k'_D < k'_L$, whereas for the non-polar amino acids $k'_L < k'_D$.

As is clear from the above reported results, the enantioselectivity (α) of the CSP containing (*S*)-PheA as selector is much higher than that observed when the chiral selector was added to the mobile phase especially for polar amino acids. For example, Dns-serine shows an α value of 5.54 at 60°C (see Table 1), whereas under the same conditions with the same selector in the mobile phase the α value was 1.2 at 25°C [18].

However, for the separation of a mixture of Dns-amino acids, better chemioselectivity was obtained with the chiral eluent in a gradient system. In this instance a mixture of eleven Dns-amino acids could be separated with baseline resolution and narrow, sharp peaks [18].

An example of the separation with the (*S*)-PheA-CSP is reported in Fig. 2. The high enantioselectivity is evident, together with a fairly good chemioselectivity, kinetic performance and high chromatographic peak symmetry.

These CSPs were also used to check the enantiomeric purity of a five-membered ring cyclic sulphonamide (γ-sultam) without any pre-derivatization procedures; an example related to the direct resolution of isothiazolidine-1,1-dioxide-3-carboxylic acid [29] is reported in Fig. 3.

It is worth noting that the reversal of the elution order of two enantiomers when moving from column A to its enantiomeric form B, and the coalescence of the two peaks when using column C, in the racemic version, constitutes

TABLE I

ENANTIOMERIC RESOLUTION OF DNS-AMINO ACIDS ON (*S*)-PheA-CSP

Column, (*S*)-PheA-CSP LiChrosorb Si 100 (5 μm) (150 × 4.0 mm I.D.); eluent, CH_3CN/0.1 *M* NH_4OAc–0.25 m*M* $Cu(OAc)_2$ (70:30, v/v); pH. = 7.52; flow-rate, 1.0 ml/min; temperature, 60°C; pressure, 700 p.s.i.; detection, UV at 254 nm.

Solute	k'_D	k'_L	α
Dns-Val	0.76	1.10	1.45
Dns-Leu	1.12	1.92	1.71
Dns-Thr	1.18	4.14	3.51
Dns-norVal	1.35	2.13	1.58
Dns-norLeu	1.42	2.05	1.44
Dns-Met	1.67	3.52	2.11
Dns-Phe	1.71	2.39	1.40
Dns-Trp	2.18	3.29	1.51
Dns-Ser	2.51	13.90	5.54
Dns-Asp	2.64	9.87	3.74
Dns-Glu	5.51	18.71	3.40

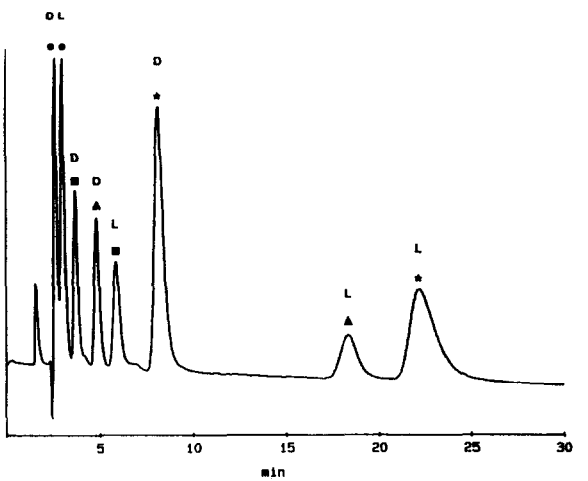

Fig. 2. Analysis of a mixture of Dns-amino acids on an (*S*)-PheA-CSP LiChrosorb Si 100 (5 μm) column (150 × 4.0 mm I.D.). Chromatographic conditions as in Table I. ● = Dns-Val; ■ = Dns-Met; ▲ = Dns-Ser; * = Dns-Glu.

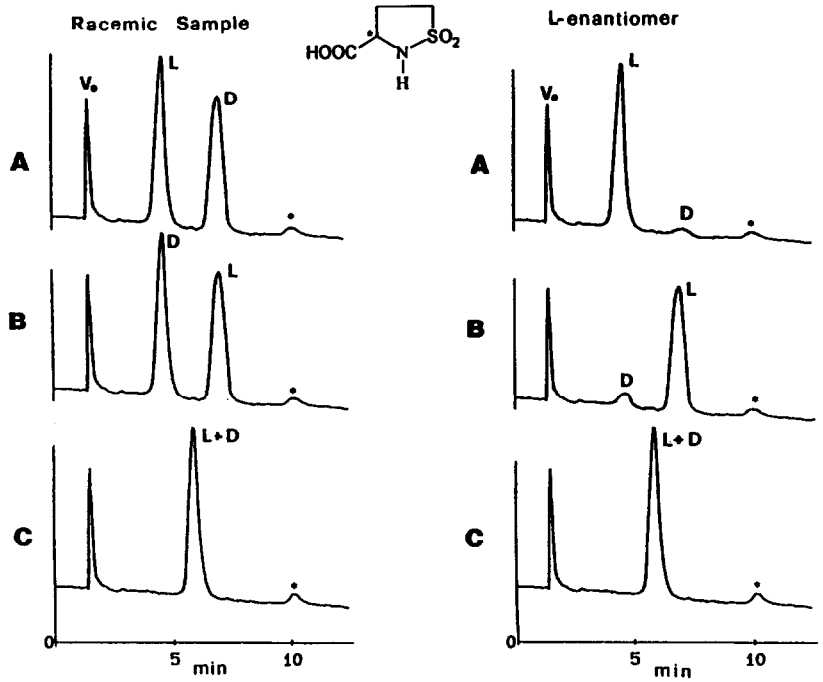

Fig. 3. Enantiomeric separation of isothiazolidine-1,1-dioxide-3-carboxylic acid (underivatized) on PheA-CSPs LiChrosorb Si 100 (5 μm) columns (150 × 4.0 mm I.D.). (A), (B), (C) indicate (*S*)-, (*R*)- and racemic versions of the CSP, respectively. Chromatographic conditions as in Table I. Detection, UV at 230 nm. Asterisks denote unknown impurities.

proof that enantiomeric separation occurs on that CSP; this is particularly useful if the enantiomers of interest are present in complex mixtures or if no racemic or scalemic (see *e.g.* ref. 37) reference sample enriched in one enantiomer is available, as often occurs with new synthetic products.

Resolution of D,L*-Dbs-amino acids*

Several enantiomeric Dbs-amino acids were completely resolved, as shown in Table II. The α values range from 1.14 to 3.34 using CH_3CN/0.1 *M* NH_4OAc–0.25m*M* $Cu(OAc)_2$ (70:30, v/v) (pH 7.52, 60°C) as the mobile phase. As already reported for Dns-amino acids on (*S*)-PheA-CSP, the D-enantiomer elutes always before the L-enantiomer, the enantioselectivity factors (α) are always high, although they are smaller than those observed for the corresponding Dns derivatives under the same experimental conditions, and the retention factor (k') seems to be lower.

Also in this instance a good chemioselectivity is present together with a kinetic performance that allows symmetrical peaks to be obtained. Hence the Dbs derivatives seem to offer a valid alternative to the Dns derivatives taking also into consideration that detection can be carried out in the visible region at 436 nm. An example of the

TABLE II

ENANTIOMERIC RESOLUTION OF Dbs-AMINO ACIDS ON (*S*)-PheA-CSP

Conditions as in Table I, except UV detection at 436 nm.

Solute	k'_D	k'_L	α
Dbs-Val	0.49	0.56	1.14
Dbs-Leu	0.68	1.03	1.51
Dbs-Ala	1.27	2.54	2.00
Dbs-Thr	1.32	4.32	3.27
Dbs-Met	1.64	2.77	1.69
Dbs-Ser	2.21	7.38	3.34
Dbs-His	2.44	2.99	1.22
Dbs-Asp	2.63	6.20	2.36
Dbs-Glu	3.84	8.85	2.30

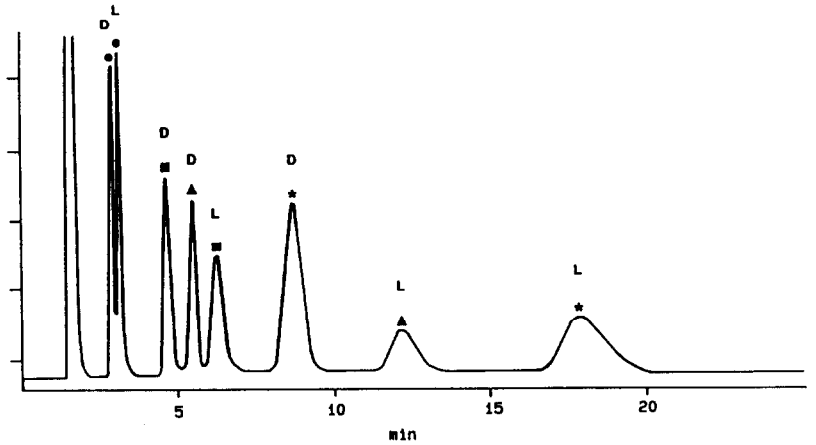

Fig. 4. Analysis of a mixture of Dbs-amino acids on an (*S*)-PheA-CSP LiChrosorb Si 100 (5 μm) column (150 × 4.0 mm I.D.). Chromatographic conditions as in Table II. ● = Dbs-Val; ■ = Dbs-Met; ▲ = Dbs-Ser, * = Dbs-Glu.

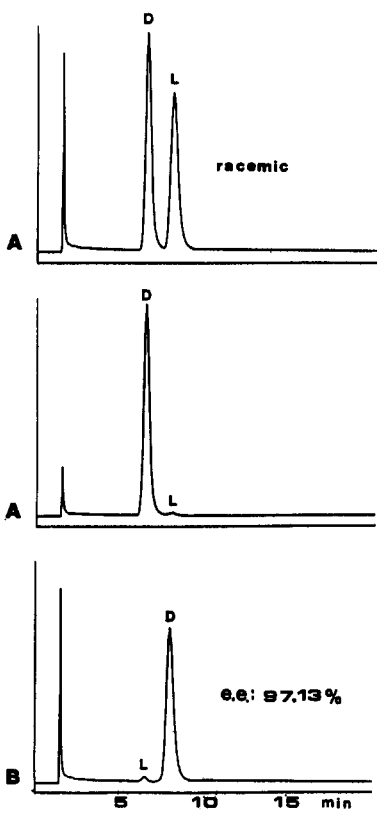

Fig. 5. Enantiomeric trace analysis of D-*p*-hydroxyphenylglycine (Dbs derivative). Column (150 × 4.0 mm I.D.) packed with (A) (*S*)-PheA and (B) (*R*)-PheA-CSP LiChrosorb Si 100 (5 μm); eluent, see Table II; flow-rate, 1.0 ml/min; temperature, 60°C; detection, UV at 436 nm. k'_{1D} = 3.27; α = 1.32; e.e. = 97.13%.

resolution of a mixture of Dbs-amino acids is reported in Fig. 4.

These CSPs were also used to check the enantiomeric purity of D-*p*-hydroxyphenylglycine obtained by a stereoselective synthesis; analytical results for a sample of D-*p*-hydroxyphenylglycine containing a small amount of the L-enantiomer (*ca.* 1.4%) are reported in Fig. 5.

Trace analysis with the appropriate chirality of the CSP

The reversal of the order of peak elution for enantiomerically enriched selectands by inverting the chirality of the selector is a useful diagnostic tool for verifying an enantiomeric separation, but it may also have an important role in quantitative enantiomer analysis [25,30–32]. Further, the precision and accuracy of steroselective chromatographic methods based on PheA-CSPs can be improved by switching from one enantiomeric form of the chiral selector to the other [both the (*R*)- and (*S*)-versions of the CSP, in addition to the racemic version, have been prepared and evaluated].

This is extremely important in the chiral chromatography when one must determine traces of an enantiomer in the presence of an excess of the other. In fact, the difficulty in detecting the more retained isomer (enantiomer) in small amounts, especially with low α values, is overcome by using a column with the opposite chirality; in this

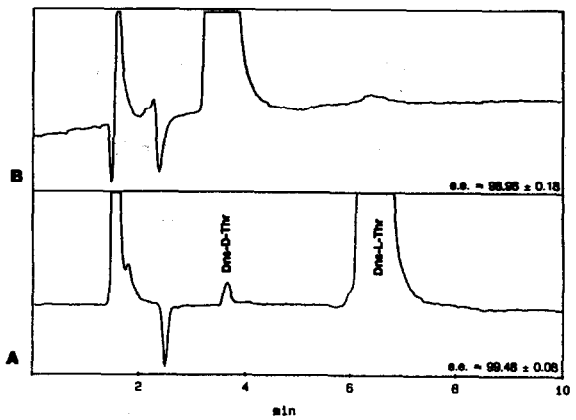

Fig. 6. Enantiomeric excess determination of Dns-L-Thr on (A) (*S*)- and (B) (*R*)-PheA-CSP LiChrosorb Si 100 (5 μm) columns (150 × 4.0 mm I.D.). Chromatographic conditions as in Table 1.

instance the impurity will be eluted first, thus making the integration easier and precise.

An example is shown in Fig. 6; six replicate injections of Dns-L-Thr containing a small amount of the D-isomer gave a mean value for the e.e. (enantiomeric excess) of 99.48 ± 0.08% using the (*S*)-PheA column (minor enantiomer eluting first) and 98.96 ± 0.18% using the (*R*)-PheA column. The detector response was linear ($r^2 > 0.99$) in the considered range of e.e.

Effect of buffer concentration

The concentration effect of the buffer (NH_4OAc) was studied with Dns-Met and Dns-Ser at pH 7.52 in a concentration range from 0.05 to 0.2 *M* at 60°C; the experimental results are reported in Table III. It is clear that the concentration effect of the buffer on the retention factor (k'_{1D}) is very moderate, whereas a slight increase in the enantioselectivity factor (α) is observed at lower buffer concentrations.

Effect of pH

The effect of pH was studied with Dns-Met and Dns-Ser in the pH range 6.0–7.57. The results are reported in Table IV. Chiral discrimination occurs throughout this pH range. As can be clearly seen, the retention factor (k'_{1D}) increases on going to lower pH (from 7.57 to 6.0), whereas the enantioselectivity factor (α) slightly decreases.

Effect of organic modifier concentration on the retention factor (k') and the enantioselectivity factor (α)

The effect of the organic modifier (CH_3CN) concentration was studied with Dns-Glu, Dns-Ser and Dns-Thr in the concentration range 30–70% (v/v) at 60°C. The results are reported in Fig. 7.

The addition of the organic modifier (acetonitrile) to the aqueous solution allowed a faster elution of both enantiomers and an increase of α for all the analyzed Dns-derivatives.

Temperature effect and thermodynamic evaluation of PheA-modified CSPs

In order to investigate the nature of the

TABLE III

EFFECT OF BUFFER CONCENTRATION ON RETENTION FACTOR (*k'*) AND ENANTIOSELECTIVITY FACTOR (α)

Column, (*S*)-PheA-CSP LiChrosorb Si 100 (5 μm) (150 × 4.0 mm I.D.); eluent, CH_3CN/NH_4OAc[a]–0.25 m*M* $Cu(OAc)_2$ (70:30, v/v); pH = 7.52; flow-rate, 1.0 ml/min; temperature, 60°C; detection, UV at 254 nm.

Solute	$k'_{1D}/k'_{1D(0)}$[a]			$\alpha/\alpha_{(0)}$[b]		
	A[c]	B[c]	C[c]	A[c]	B[c]	C[c]
Dns-Met	0.68	1.00	0.95	1.08	1.00	0.90
Dns-Ser	0.70	1.00	0.98	1.09	1.00	0.95

[a] $k'_{1D}/k'_{1D(0)}$ = relative retention factor; $k'_{1D(0)}$ = retention factor of the D-enantiomer at [NH_4OAc] = 0.10 *M*.
[b] $\alpha/\alpha_{(0)}$ = relative enantioselectivity factor; $\alpha_{(0)}$ = enantioselectivity factor at [NH_4OAc] = 0.10 *M*.
[c] [NH_4OAc] = (A) 0.05, (B) 0.10 and (C) 0.20 *M*.

TABLE IV

EFFECT OF MOBILE PHASE pH ON THE RETENTION FACTOR (k') AND THE ENANTIOSELECTIVITY FACTOR (α)

Column, (*S*)-PheA-CSP LiChrosorb Si 100 (5 μm) (150 × 4.0 mm I.D.); eluent, CH_3CN/0.1 *M* NH_4OAc–0.25 m*M* $Cu(OAc)_2$ (70:30, v/v); flow-rate, 1.0 ml/min; temperature, 60°C; detection, UV at 254 nm.

Solute	$k'_{1D}/k'_{1D(0)}$ [a]			$\alpha/\alpha_{(0)}$ [b]		
	A[c]	B[c]	C[c]	A[c]	B[c]	C[c]
Dns-Met	4.55	4.45	1.00	0.84	0.88	1.00
Dns-Ser	4.73	4.06	1.00	0.89	0.88	1.00

[a] $k'_{1D}/k'_{1D(0)}$ = relative retention factor; $k'_{1D(0)}$ = retention factor of the D-enantiomer at pH 7.57.
[b] $\alpha/\alpha_{(0)}$ = relative enantioselectivity factor; $\alpha_{(0)}$ = enantioselective factor at pH 7.57.
[c] pH = (A) 6.0, (B) 6.5 and (C) 7.57.

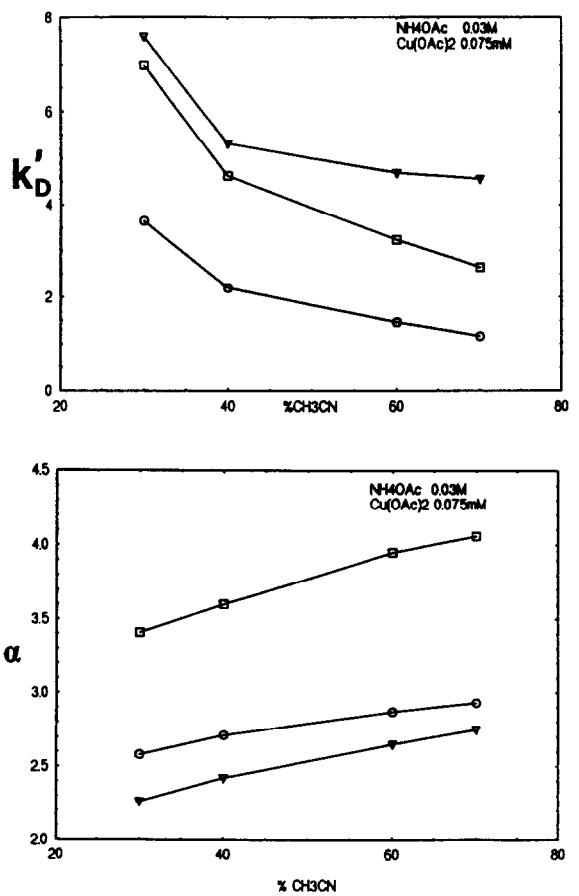

Fig. 7. Influence of organic modifier concentration on the retention factor (k') and the enantioselectivity factor (α). Column, (*S*)-PheA-CSP LiChrosorb Si 100 (5 μm) (150 × 4.0 mm I.D.); temperature, 60°C. ▽ = Dns-Glu; □ = Dns-Ser; ○ = Dns-Thr.

interactions involved in the discrimination process, the temperature effect on both the retention factor, k', and the enantioselectivity factor, α, was studied [33]. Several chromatograms for the resolution of the racemic mixture of Dns-Met, carried out at different temperatures (30–60°C) on an (*S*)-PheA column, are shown in Fig. 8; chiral discrimination occurs in the temperature range examined.

The results obtained clearly show that at the lower end of the temperature range investigated, the retention factors (k') of both isomers increase while a certain amount of peak broadening occurs, which may be due to a slow mass-transfer process occurring in the above temperature range or to slower kinetics of complexation–decomplexation for the copper complex.

The effect of the temperature [34] on k' and α, is described by the equations

$$\ln k'_i = -\frac{\Delta H^0_i}{RT} + \frac{\Delta S^0_i}{R} + \ln \phi \quad (1)$$

$$\ln \frac{k'_i}{k'_j} = \ln \alpha = -\frac{\Delta\Delta H^0_{ij}}{RT} + \frac{\Delta\Delta S^0_{ij}}{R} \quad (2)$$

where ΔH^0 and ΔS^0 are the enthalpy and the entropy changes, respectively, associated with the analyte retention process, and ϕ is the phase ratio; the subscripts i and j refer to the enantiomeric form of a generic racemic solute. Both equations predict a linear inverse relationship between ln k' or ln α and temperature.

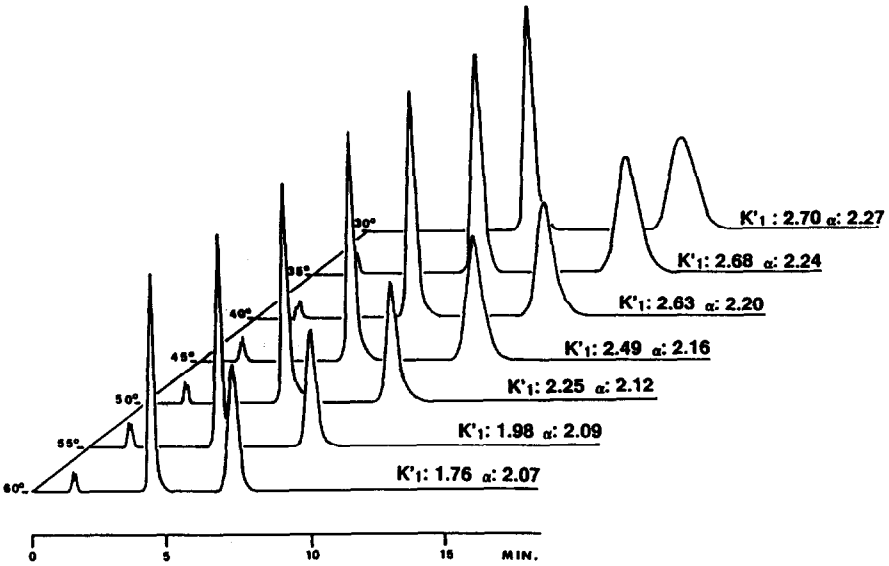

Fig. 8. Effect of temperature on the retention factor (k') and the enantioselectivity factor (α) for D,L-Dns-Met. Column, (*S*)-PheA-CSP LiChrosorb Si 100 (5 μm) (150 × 4.0 mm I.D.); eluent, see Table I; flow-rate, 1.0 ml/min; temperature, from 30°C (top) to 60°C (bottom) in 5°C increments.

The values of the thermodynamic parameters were derived from experiments carried out at different temperatures ranging from 20 to 70°C for Dns-Ser, Dbs-Ser, Dns-Met, Dbs-Met, Dns-Thr and Dbs-Thr. No decomposition was observed in this range of temperature; in experiments carried out from 24 to 70°C on an (*S*)-PheA column, we measured the peaks area of racemic Dns-Leu and Dns-Thr; the variations were <1% for both solutes.

Typical graphs of these relationships (Van 't Hoff plots) are reported in Figs. 9A and B and 10. The results clearly show that for all the separations carried out, a very high linearity is observed ($r^2 > 0.99$). The thermodynamic parameters obtained are given in Table V.

$\Delta\Delta H^0$ and $\Delta\Delta S^0$ have the same sign for all the amino acid derivatives examined (Dns and Dbs); the enthalpic and entropic terms have opposite effect on the recognition process, as is usually observed in enantioselective HPLC on monomeric phases. The unfavourable $\Delta\Delta S^0$ term essentially arises from the loss of degrees of freedom experienced by the most retained enantiomer, which forms a more tightly bound complex with the chiral selector. However, other processes contribute to the magnitude (and sign) of $\Delta\Delta S^0$ (*e.g.*, desolvation of the analytes and of the chiral selector and resolvation on complexation, and coordination of the ligands around the central Cu^{2+} ion) and for some of the amino acids these different contributions to the entropic term cancel each other, resulting in $\Delta\Delta S^0$ close to zero (Dns-Ser, Dns-Thr). Under such circumstances, there is little variation in α with temperature and chromatography can be conveniently carried out above room temperature, taking advantage of the increased efficiency obtained without significantly affecting the enantioselectivity. The relative value of $\Delta\Delta H^0$, $\Delta\Delta S^0$ and the high enantioselectivity factor (α) allow separations at 60°C, thus obtaining a good compromise between efficiency, CSP chemical stability and selectivity.

Chiral recognition mechanism

In order to discuss the interactions responsible for chiral discrimination, it is necessary to know the structure of the copper complex formed on the phase. In the pH range considered here in aqueous solution phenylalaninamide forms the species CuL^{2+}, $CuLH^{+}_{-1}$, $CuL_2H^{+}_{-1}$ and CuL_2H_{-2}, as shown by potentiometry and spectroscopy (absorption and CD) [20]. On account of the concentration of the ligand on the phase (CSP), it is most feasible that the species

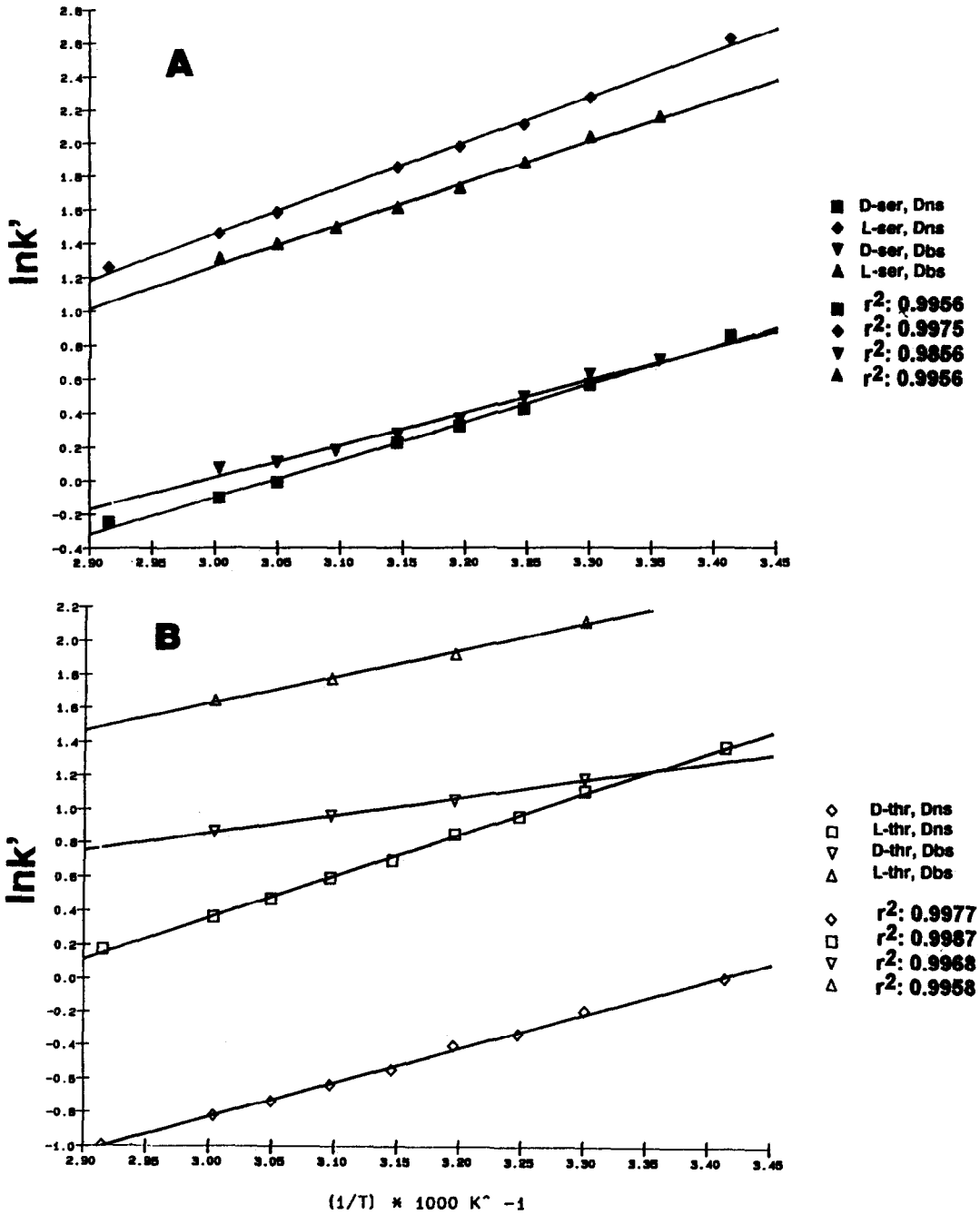

Fig. 9. Linear inverse relationship between ln k' and temperature for Dns and Dbs derivatives of (A) serine and (B) threonine. Chromatographic conditions as in Table I (Dns) and Table II (Dbs).

$CuLH^{+}_{-1}$ is present at pH 7.0–7.5. However, to obtain a closer model of the phase, we synthesized the ligands (*S*, *R*)- and (*S*, *S*)-N^2-(2-hydroxypropyl)phenylalaninamide and studied the presence of the species as a function of pH [35]. On the phase at pH 7.5, a positively charged

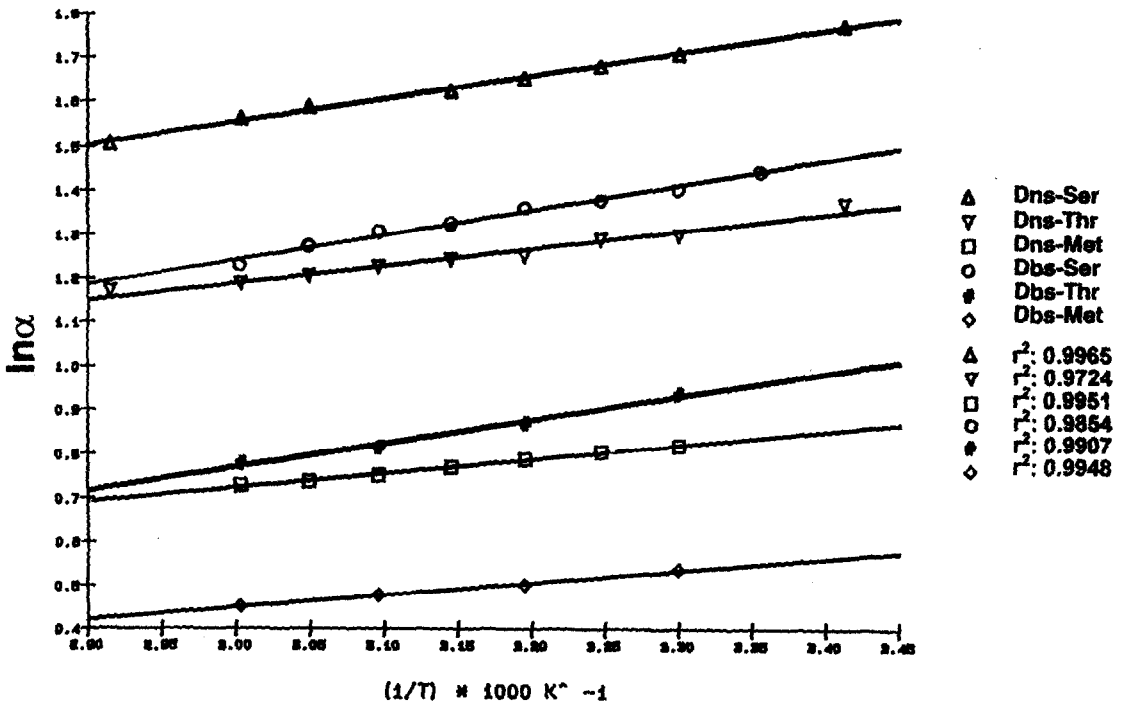

Fig. 10. Linear inverse relationship between ln α and temperature for Dns and Dbs derivatives of serine, threonine and methionine. Column, (*S*)-PheA-CSP LiChrosorb Si 100 (5 μm) (150 × 4.0 mm I.D.); eluent, see Table I; flow-rate, 1.0 ml/min; detection, UV at 254 and 436 nm.

species of the type $CuLH^{+}_{-1}$ with the hydroxy group at the apical position is most likely present. The Dns-amino acid is forced to approach the bound selector in an allowed position, with the bulky Dns-group *cis* relative to the amide group. In this way the side-chain of the Dns-amino acid gives a repulsive interaction with the phase, in particular with the hydroxy group.

In contrast, in the eluent, the mixed complex may assume the most stable *trans* configuration, and the elution order reflects a preferential interaction of the lipophylic dansyl group with

TABLE V

THERMODYNAMIC DATA

Solute	Eluent[a]	R'HN*CHRCONH$_2$ [b]	$-\Delta\Delta H°$ (cal/mol)	$-\Delta\Delta S°$ (cal/mol · K)	$-\Delta\Delta G$(25°C) (cal/mol)
Dns-Ser	A	R = $-CH_2OH$	1039	0.03	1030
Dbs-Ser	B		1139	0.95	856
Dns-Met	A	R = $-CH_2CH_2SCH_3$	649	0.51	497
Dbs-Met	A		572	0.82	328
Dns-Thr	A	R = $-CH(CH_3)OH$	804	0.05	789
Dbs-Thr	A		1080	1.71	570

[a] (A) See Tables I and II; (B) CH_3CN–[CH_3OH/0.083 *M* NH_4OAc–0.25 m*M* $Cu(OAc)_2$ (1:2, v/v)] (75:25, v/v).
[b] R' = Dns or Dbs.

the C_{18} column, and a hydrophobic–hydrophilic effect of the amino acid side-chain, producing an opposite elution order for polar and apolar amino acids [36]. Finally, we can assert that it is misleading to take into consideration the stability constant of ternary complexes formed in solution with a soluble selector closely related to the CSP as a model for the enantioselectivity observed on the HPLC column. The type and length of the spacer used to connect the selector to the solid support determine the geometry of the mixed complex to be formed and the repulsive interactions with this moiety becomes fundamental to the recognition process.

CONCLUSIONS

We have provided a simple method for the preparation of chiral stationary phases (CSPs) for LEC for the direct enantiomeric resolution of Dns and Dbs derivatives of amino acids. The enantioselectivity factors obtained are always very high; the molecular recognition mechanism is under enthalpic control under the experimental conditions; the entropic contribution is almost always very small and sometimes near zero. In conclusion, the thermodynamic parameters permit operation at fairly high temperature (60°C) without lowering the enantioselectivity and taking advantage of the kinetic performance of the chromatographic system.

These CSPs were also used successfully in the enantiomeric resolution of a five-membered ring cyclic sulphonamide (γ-sultam). The results obtained showed good chemical stability of the CSP and considerable reproducibility.

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