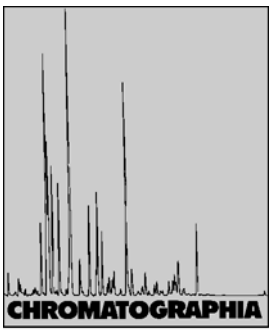
Chiral Ligand-Exchange Chromatography on an RP HPLC Column Coated with a New Chiral Selector Derived from L-Spinacine



2002, 55, 301–306

M. Remelli* / D. Trombin / C. Conato

Department of Chemistry, University of Ferrara, via L. Borsari 46, 44100 Ferrara, Italy; E-Mail: rmm@unife.it

Key Words

Column liquid chromatography
Chiral separations
Ligand exchange
N^{τ}-*n*-decyl-L-spinacine
Amino acids

Summary

A commercial reversed-phase (RP) C_{18} HPLC column has been dynamically coated with the chiral selector N^{τ}-*n*-decyl-L-spinacine and then loaded with copper(II) ions. Several racemic mixtures of underivatized amino acids and oligopeptides were resolved on the column by chiral ligand-exchange chromatography. The most important experimental conditions affecting column efficiency, retention, and selectivity (temperature and mobile phase flow rate and composition) were extensively investigated.

Introduction

The chiral separation of enantiomeric compounds has attracted increasing attention in recent years, mostly because enantiomers often have different effects on living organisms. The most efficient chiral separation methods, for both analytical and preparative purposes, are those based on chromatography [1–5]. Successful separation of underivatized amino acid and oligopeptide enantiomers has been achieved with chiral ligand-exchange chromatography (CLEC) [6]. This technique was first introduced by Rogozhin and Davankov who used a chiral stationary phase (CSP) derived from L-proline [7]. Metal complexation for enantiomer resolution by use of a chiral mobile phase (CMP) additive was pioneered by Karger et al. [8–10] CLEC is based on the formation of labile ternary metal complexes in the mobile or stationary phase – one of the ligands is a suitable chiral selector whereas the second is one of the components of the sample mixture. A suitable concentration of the complexing metal ion is present in both the mobile and stationary phases. Thus, enantiomeric sample ligands can form diastereomeric complexes with the metal ion and the chiral selector and even small differences in stability can lead to enantiomer separation. Several chiral selectors, often appropriate L-amino acid derivatives, have been used in CLEC, as chiral modifiers of both mobile and stationary phases [6, 11–16]. Separation of amino acid enantiomers by ligand-exchange micellar electrokinetic chromatography has also recently been reported [17].

We have previously reported the synthesis of N^{τ}-decyl-L-histidine, His(τ-dec) [11, 18, 19]. This chiral selector can be strongly adsorbed by the hydrophobic stationary phases used for reversed-phase chromatography, fully preserving the complexing properties of His [20]. The chiral modification of RP HPTLC plates [18] and RP HPLC columns [11, 19] with His(τ-dec), by means of a dynamic coating technique, showed that this chiral selector enables good resolution of racemic mixtures of underivatized amino acid enantiomers.

This paper describes the use of a new selector, N^{τ}-*n*-decyl-L-spinacine, Spi(τ-dec), (Figure 1) for CLEC. Spi [21, 22] is a condensation product of His with formaldehyde (FA) and contains a methylene bridge between the amino group and the imidazole ring. A commercial ODS column has been modified with this chiral selector, by means of the dynamic coating technique mentioned above. The retention, selectivity, and efficiency of the new column toward racemic mixtures of un-

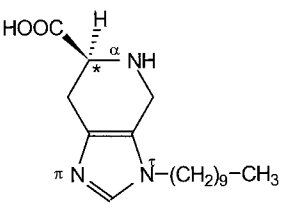


Figure 1. The structure of the chiral selector N^{τ}-*n*-decyl-L-spinacine.

0009-5893/00/02 301-06 $ 03.00/0

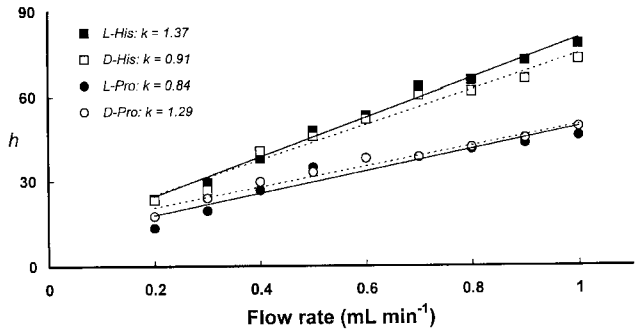


Figure 2. Column efficiency, reported as reduced plate height, as a function of mobile-phase velocity. The mobile phase was 10^{-2} M acetate buffer, pH 4, containing 2×10^{-2} M copper acetate at T = 30 °C. The reported k values were measured at 1 mL min^{-1} (Table III).

derivatized amino acids and oligopeptides have been investigated. The effect of the most important experimental conditions on the chromatographic results is discussed.

Experimental

Materials

HPLC-grade methanol (MeOH) and acetonitrile (ACN) and tetra-distilled water were used as solvents. Copper(II) acetate, potassium phosphate, and acetic acid (Carlo Erba) used to prepare mobile phases and amino acids (Aldrich and Sigma) were high-purity products. The samples were injected as 0.01–0.1% aqueous solutions. Mobile phase pH was adjusted by addition of suitable amounts of a standard KOH or $HClO_4$ solutions, under potentiometric control. The synthesis and characterization of the chiral selector Spi(τ-dec) are described elsewhere [23].

Chromatographic Equipment

HPLC was performed with a Waters model 600 multi-solvent delivery system equipped with a Rheodyne model 7010 injection valve (20-µL sample loop) and a Waters model 996 photodiode-array detector (8-µL cell) coupled with a Digital Venturis FX5133s personal computer driven by Waters Millennium 32 software. The wavelength-range employed for detection was 200–300 nm.

A commercial Symmetry (Waters) stainless-steel analytical column (4.6 mm × 250 mm) was used for RP HPLC. The packing was 5-µm spherical particles of end-capped octadecyl silica (ODS) with a surface area of 344 m^2 g^{-1}, a mean pore diameter of 91 Å, and a packing weight of 3.3 g, giving a total stationary-phase surface area of 1153 m^2. A typical loading was 19.8% C (3.2 µmol m^{-2} alkyl chains). The column dead volume, measured by using KNO_3 as a marker, was 2.00 mL.

The column was thermostatted by means of a column jacket connected to a Haake model F3C circulating thermostat.

Chiral Column Preparation

A solution (0.9% w/v) of Spi(τ-dec) in 60:40 MeOH-H_2O buffered at pH* 7.2 with 10^{-2} M phosphate buffer was passed through the column (previously equilibrated with the solvent and thermostatted at 30 °C) at 0.2 mL min^{-1}, in frontal mode, until the breakthrough step was observed and the detector response proved stable. The chiral-selector loading, calculated from the Spi(τ-dec) breakthrough time, measured graphically and corrected for the column dead-volume, was 0.63 mmol. This was equivalent to a coverage of 0.55 µmol m^{-2} of chiral selector, approximately one Spi(τ-dec) molecule for six alkyl chains. The average surface area available for each chiral-selector molecule is 300 Å^2. The modified column was washed with aqueous phosphate buffer (10^{-2} M, pH 6) and with acetate buffer (10^{-2} M, pH 5) and then treated with the copper(II) acetate solution used as mobile phase ([$Cu(Ac)_2$] = 10^{-3} M in acetate buffer 10^{-2} M, pH 5). From the observed breakthrough step for copper it was possible to calculate that the amount of Cu(II) ion loaded on the chiral column was 0.35 mmol. The ratio chiral selector/copper ion was, therefore, 1.8, suggesting that the chiral layer on the RP support comprises mainly bis complexes in which a Cu(II) ion is chelated by two immobilised Spi(τ-Dec) ligands.

The stability of the chiral layer was checked by monitoring the chromatographic behaviour of the D/L-Pro pair, at 30 °C, using a 2×10^{-3} M solution of copper acetate in acetate buffer (10^{-2} M, pH 5) as mobile phase. After six months of intense use both retention (k) and enantioselectivity (α) had decreased, but by less then 10% of their original values. Similar behaviour was also observed for the other samples for which the order of elution never changed. Chromatographic data reported in any table in this paper were measured within a few days during which column performance remained constant.

Chromatographic Measurements

Potassium nitrate was used as column void-volume marker (on the unmodified column). The capacity ratio (k) values were computed by measuring the retention time (t_R) at the peak maximum. Column efficiency was calculated as the reduced plate height (h) from the equation:

$$h = 0.18\, L (w_h/t_R)^2/d_p = H/d_p$$

where L is the column length, w_h the peak width measured at half peak height, d_p the stationary phase particle diameter, and H the plate height; h is a pure number. Enantioselectivity, α, was always computed as k_D/k_L, where the subscripts refer to the enantiomers. The order of elution for a racemic sample was checked by injection of single enantiomers. The peak asymmetry factor, A_s, is the ratio of the right half-width at 1/10 of peak height to the corresponding left half-width.

Results and Discussion

The most important experimental conditions affecting both sample retention and resolution were investigated. More than twenty racemic mixtures of amino acids and oligopeptides were used as enantiomeric samples; it was rarely impossible to achieve good resolution.

Flow-rate

Figure 2 shows the dependence of column efficiency, calculated for D/L-His and D/L-Pro, on mobile-phase flow-rate. Plate height decreased linearly when the mobile phase velocity was reduced, and at 0.2 mL min^{-1} a minimum had not yet been reached. This trend was similar to that already observed for use of His(τ-Dec) as

chiral selector [11] and supports the hypothesis that the relatively low efficiency is mainly because of slow exchange kinetics [24, 25]. The slope of the HETP curve depends on the type of sample, and is larger for the D/L-His pair, possibly because of the tridentate character of this ligand [20]. In contrast, the efficiency curve is approximately the same for both enantiomers of each pair, implying the phase-exchange kinetics are similar.

Copper(II) Concentration

Increasing the concentration of copper in the mobile phase from 5×10^{-5} to 1×10^{-3} M resulted in an initial increase and then a decrease in solute retention; this reached a maximum for the intermediate $[Cu^{2+}]$ value of 2×10^{-4} M, the only exception being D/L-Pro (Table I). This behaviour can be understood by observing that the concentration of copper in the mobile phase plays a double role in this chromatographic system. First, when the concentration is low its increase causes a corresponding increase in the number of active sites on the stationary phase (i. e. the copper complexes with the fixed chiral selector). As a consequence all the solutes are more retained. Then, when the stationary phase is completely saturated with copper, a further increase in the Cu(II) ion content of the mobile phase shifts the equilibrium between fixed and mobile complexes in favour of the latter – in other words the mobile phase becomes 'stronger' and solute retention decreases. It is worth noting that despite the large retention changes the enantioselectivity is mostly unaffected.

The behaviour of His is peculiar. First, the order of elution of the enantiomers is the opposite of that for the other amino acids. This is probably because His preferentially binds the copper ion differently from the other amino acids [20], i. e. by means of its amino and imidazole nitrogen atoms (*histamine-like*) instead of the amino nitrogen and carboxylic oxygen atoms (*glycine-like*). This difference has important consequences on the structure and the stability of the corresponding ternary complexes [20] and explains the particular chromatographic behaviour of this amino acid. The retention of both enantiomers decreases, moreover, when $[Cu^{2+}]$ is increased from 5×10^{-5} to 2×10^{-4} M; the same happens for Asn. This behaviour might be because the partial tridentate character of both ligands towards the copper ion [20, 26] renders their mobile binary complexes particularly stable and sensitive to the concentration of copper in the mobile phase.

Finally, Table II shows that both column efficiency and the peak symmetry improve for D/L-Pro when $[Cu^{2+}]$ is increased from 5×10^{-5} to 2×10^{-4} M. A slightly lower h value is observed for the less-retained enantiomer.

Table I. Effect on retention and selectivity of the concentration of copper acetate in the mobile-phase. The mobile phase was 10^{-2} M acetate buffer, pH 5, at T = 30 °C and a flow rate of 1 mL min^{-1}.

Sample	$[Cu^{2+}] = 1 \times 10^{-3}$ M		$[Cu^{2+}] = 2 \times 10^{-4}$ M		$[Cu^{2+}] = 5 \times 10^{-5}$ M	
	k	α	k	α	k	α
DL-Ala	4.17	1	4.22	1	1.89	1
L-Asn			9.31	1.07	10.2	1.04
D-Asn			9.98		10.6	
DL-Gln			6.57	1	5.13	1
L-His			8.00	0.71	12.1	0.66
D-His			5.64		7.99	
L-Leu			27.1	1.32	21.7	1.32
D-Leu			35.7		28.6	
DL-Lys	0.52	1	0.56	1	0.32	1
L-Met			25.5	1.34	17.5	1.47
D-Met			34.1		25.7	
DL-Orn	0.56	1	0.68	1	0.40	1
L-Pro	5.53	1.53	5.23	1.52	3.26	1.71
D-Pro	8.48		7.96		5.58	
L-Ser	5.48	1.10	7.52	1.09	5.30	1.07
D-Ser	6.00		8.21		5.69	
DL-Thr	5.53	1	8.13	1	8.20	1
L-nVal			11.7	1.27	5.80	1.28
D-nVal			14.8		7.39	
L-Val	10.59	1.37	12.9	1.41	5.59	1.37
D-Val	14.47		18.2		7.65	

Table II. Effect of some experimental conditions on column efficiency and peak asymmetry. The mobile phase was aqueous copper acetate-10^{-2} M acetate buffer at a flow rate of 1 mL min^{-1}.

$[Cu^{2+}]$ (M $\times 10^3$)	pH	T (°C)	Sample	k	h	A_s
0.05	5.0	30	L-Pro	2.58	51.7	1.5
			D-Pro	5.92	72.0	1.6
0.20			L-Pro	5.05	43.6	1.5
			D-Pro	7.56	45.3	1.6
0.20	4.0	30	L-Pro	0.85	43.3	2.1
			D-Pro	1.32	58.2	1.8
	4.5		L-Pro	2.31	62.6	2.0
			D-Pro	3.70	–	–
	5.0		L-Pro	5.05	43.6	1.5
			D-Pro	7.56	45.3	1.6
	5.5		L-Pro	10.1	46.9	1.7
			D-Pro	15.8	63.1	2.0
0.20	5.0	15	L-Pro	6.24	74.6	1.3
			D-Pro	9.21	66.6	1.4
		30	L-Pro	5.05	43.6	1.5
			D-Pro	7.56	45.3	1.6
		45	L-Pro	5.47	31.8	1.5
			D-Pro	7.89	34.3	1.5
		60	L-Pro	5.17	29.8	1.6
			D-Pro	7.31	28.6	1.4

Mobile Phase pH

Mobile phase pH is one of the most important conditions affecting the positions of binary and ternary complex-formation equilibria, and then amino acid retention. It is well known [20] that, at neutral or alkaline pH, ternary (stationary) complexes are favoured over binary (mobile) species. Table III shows that all the samples are increasingly retained as pH increases; this change is less marked for amino acids with a basic side-chain. Mobile-phase pH has

Table III. Effect of mobile-phase pH on retention and selectivity. The mobile phase was 10^{-2} M acetate buffer containing 2×10^{-4} M aqueous copper acetate, at $T = 30$ °C and a flow rate of 1 mL min^{-1}.

Sample	pH 4		pH 4.5		pH 5		pH 5.5	
	k	α	k	α	k	α	k	α
DL-Ala	0.77	1	1.69	1	4.19	1	6.17	1
DL-Arg	0.01	1	0.23	1			2.55	1
L-Asn	2.39	1	5.56	1	9.31	1.07	12.1	1.08
D-Asn					9.98		13.0	
DL-Asp	48.3	1.19			93.9	1.22		
	57.3				114			
DL-Gln			3.23	1	6.57	1	10.9	1
DL-Glu	16.9	1.10			60.5	1.11		
	18.6				67.0			
L-His	1.37	0.66	4.97	0.70	8.00	0.71	9.89	0.73
D-His	0.91		3.47		5.64		7.20	
L-Leu	5.04	1.27	14.3	1.28	27.1	1.32		
D-Leu	6.40		18.4		35.7			
DL-Lys	0.00	1	0.13	1	0.56	1	1.68	1
L-Met	4.82	1.42	14.4	1.36	25.5	1.33	41.0	1.29
D-Met	6.82		19.6		34.1		52.9	
DL-Orn	0.00	1	0.15	1	0.68	1	1.69	1
L-Pro	0.84	1.53	2.30	1.61	5.23	1.52	10.1	1.56
D-Pro	1.29		3.70		7.96		15.8	
D-Ser	1.36	1	3.52	1.08	7.52	1.09	9.73	1.11
L-Ser			3.79		8.21		10.8	
DL-Thr	2.05	1	4.52	1	8.13	1	11.7	1
L-Val	1.67	1.41	5.42	1.41	12.9	1.41	20.7	1.39
D-Val	2.36		7.67		18.2		28.9	

Table IV. Effect on retention and selectivity of addition of ammonium acetate to the mobile phase. The mobile phase was 10^{-2} M acetate buffer containing 2×10^{-4} M aqueous copper acetate, at $T = 30$ °C and a flow rate of 1 mL min^{-1}.

Sample	Ammonium acetate 10^{-3} M		Ammonium acetate 10^{-2} M			
	pH 6.0		pH 6.0		pH 6.5	
	k	α	k	α	k	α
DL-Ala	6.26	1	6.06	1	9.53	1
DL-Arg	2.85	1	4.59	1	5.14	1
DL-Asn	6.99	1	7.65	1	13.7	1
DL-Asp	104	1.19	28.7	1.17		
	124		33.6			
L-His	6.07	0.72	9.23	0.79	12.7	0.78
D-His	4.36		7.28		9.92	
DL-Gln	6.98	1	7.35	1.08	11.6	1
			7.95			
DL-Glu	94.1	1.14	28.3	1.16		
	107		32.8			
DL-Lys	1.68	1	2.65	1	3.57	1
DL-Orn	1.53	1	2.51	1	3.45	1
L-Pro	10.5	1.67	13.5	1.60	16.2	1.52
D-Pro	17.5		21.6		24.6	
L-Ser	6.13	1	6.61	1.09	10.7	1.10
D-Ser			7.23		11.7	
DL-Thr	6.04	1	7.46	1	12.3	1
L-Val	17.8	1.52	25.0	1.42	28.2	1.36
D-Val	27.1		35.6		38.3	

only a moderate and ambiguous effect on enantioselectivity – a minimum pH is necessary to achieve enantiomer resolution for a few samples only (Ser and Asn). In contrast, for the most retained samples the minimum pH (4) enables very good resolution in a reduced run time. Unfortunately, pH < 4 are not suggested, because of the high solubility of the chiral selector.

Both column efficiency and peak asymmetry depend only slightly on mobile phase pH, as is apparent from the data for D/L-Pro (Table II).

A maximum pH of 5.5 should be employed with acetate buffer, to avoid precipitation of Cu(II) hydroxide.

Ammonium Acetate Buffer

If the acetic acid-potassium acetate buffer is substituted with ammonium acetate the formation of Cu(II)-ammonia complexes prevents precipitation. Under these conditions, therefore, it proved possible to explore higher pH values. The results obtained by use of different concentrations of ammonium acetate, at pH 6.0 and 6.5, are shown in Table IV.

Comparison of the data reported in Tables III and IV reveals that two opposite effects determine sample retention. First, as pH is increased the formation of ternary stationary complexes is favoured and sample retention increases, as already observed above. This behaviour is the same for each sample when pH changes from 6.0 to 6.5 (Table IV, columns 2 and 3) with the same buffer concentration. The corresponding enantioselectivity is mostly unaffected. Free ammonia, which is present in the mobile phase, competes for copper with the sample ligands, with ambiguous effects on sample retention, when the pH is increased from 5.5 (acetate buffer) to 6.0 (ammonium acetate). Basic and aliphatic amino acids are, simply, increasingly retained when pH increases. The other amino acids seem instead to be more sensitive to the presence of ammonia, which works as a 'scavenger', removing the samples from the stationary phase sites and pushing them towards the end of the column. As a consequence, retention decreases when the buffer is changed, even though the pH is increased from 5.5 to 6.0. This effect is especially relevant for the acidic amino acids (Asp and Glu), for which dramatic loss of retention is observed when the ammonium acetate concentration is increased from 10^{-3} to 10^{-2} mol dm^{-3}. The buffer concentration not only affects the ionic strength and buffer capacity of the mobile phase (and thus the 'local' pH inside the sample zone) but also the ammonia concentration. In contrast, the other samples are more retained (but less resolved) at the highest ammonium acetate concentration.

Column Temperature

Figure 3 shows that increasing the column temperature from 15 to 60 °C has different effects on peak retention. Usually the higher the temperature the lower are the k values. In contrast, the retention of the aliphatic amino acids (n-Val, Val and Leu)

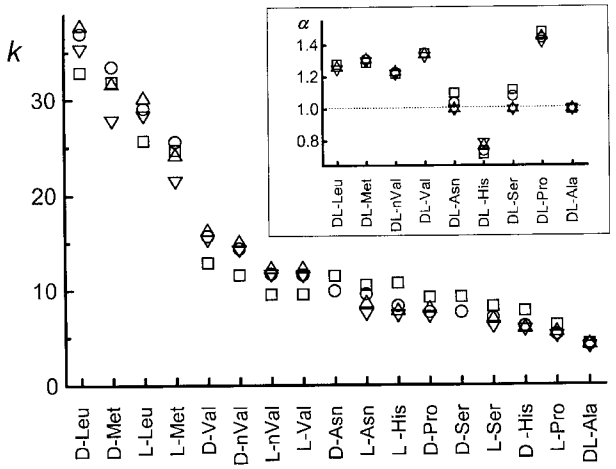


Figure 3. Effect of column temperature on retention and selectivity. The mobile phase was 10^{-2} M acetate buffer, pH 5, containing 2×10^{-2} M copper acetate, and the flow rate was 1 mL min^{-1}. □, 15 °C; ○, 30 °C; △, 45 °C; ▽, 60 °C.

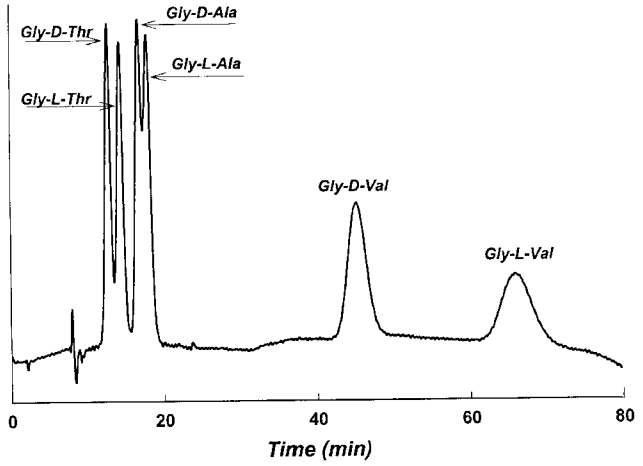


Figure 4. Separation of the enantiomers of underivatized dipeptides on an RP_{18} column treated with Spi(7-Dec). The mobile phase was 10^{-2} M acetate buffer, pH 5, containing 2×10^{-2} M copper acetate, at $T = 30$ °C and a flow rate of 1 mL min^{-1}; the detection wavelength was 230 nm.

and Met is maximum at 30–45 °C. Complete investigation of the corresponding partition isotherms would be necessary if this behaviour is to be completely understood, but this is beyond the scope of this work. Enantioselectivity is invariably reduced by increasing the temperature, although no dramatic changes are observed. Column efficiency is always improved by increasing the temperature, because of the faster ligand-exchange kinetics. Asymmetry usually seems to be unaffected (Table II).

Type and Amount of Organic Modifier

Table V shows that the presence of an organic solvent in the mobile phase, even at low levels, has a significant affect on sample retention and generally leads to loss of column selectivity. The organic modifier weakens the hydrophobic interactions between the sample and the ODS support. Such interactions are the main sources of retention for hydrophobic amino acids, the retention of which is less in the presence of an organic solvent. In contrast, for both acidic and basic samples a less polar mobile phase seems to favour interaction with the stationary-phase sites, leading to a noticeable increase in the retention of both enantiomers. The effect of the two different organic modifiers is almost the same. If the amount of organic solvent is too high column stability can be compromised.

Table V. Effect on retention and selectivity of addition of organic modifier to the mobile phase. The mobile phase was 10^{-2} M acetate buffer, pH* 5, containing 2×10^{-4} M aqueous copper acetate, at $T = 30$ °C and a flow rate of 1 mL min^{-1}.

Sample	EtOH 5%		ACN 5%	
	k	α	k	α
DL-Ala	4.03	1	3.75	1
DL-Asn	9.23	1	8.28	1
DL-Asp	112 139	1.25	95.7 113	1.19
DL-Gln	6.05	1	5.48	1
DL-Glu	69.7 75.2	1.08	62.1 67.1	1.08
D-His L-His	6.50 8.62	0.75	5.50 7.43	0.74
L-Leu D-Leu	17.8 20.0	1.13	19.9 22.0	1.10
DL-Lys	1.57	1	1.27	1
L-Met D-Met	15.9 18.3	1.15	17.4 19.9	1.14
DL-Ser	7.03	1	6.37	1
DL-Thr	7.22	1	6.69	1
L-Val D-Val	8.18 9.44	1.15	8.55 9.67	1.13

Table VI. Retention and selectivity for some dipeptides containing glycine. The mobile phase was 10^{-2} M acetate buffer, pH 5, containing 2×10^{-4} M aqueous copper acetate, at $T = 30$ °C and a flow rate of 1 mL min^{-1}.

Sample	k	α
Gly-DL-Asn	5.87	1
Gly-D-Thr Gly-L-Thr	5.40 6.20	1.15
Gly-D-Ala Gly-L-Ala	7.06 8.99	1.27
D-Ala-Gly L-Ala-Gly	7.42 7.98	1.08
Gly-D-Val Gly-L-Val	21.6 32.0	1.48
DL-Leu-Gly	113 132	1.16

Separation of the Enantiomers of Dipeptides

The chiral column used in this work proved useful for the analysis of dipeptides containing Gly as the first or second residue. The best results are reported in Table VI, and a chromatogram is shown in Figure 4. The Gly-dipeptides are generally more retained than the corresponding amino acids, and are well resolved, except for Gly-Asn. The order of elution is always D before L, the opposite of that found for the simple amino acids. The retention of Gly-Ala and Ala-Gly is very similar, although the former is better resolved. The dipeptides investigated seem to behave as substituted glycines – the first residue binds the metal ion by means of its terminal amino nitrogen and the carbonyl oxygen of the peptide bond (NO coordi-

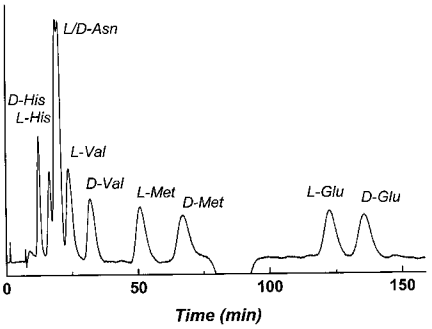


Figure 5. Separation of the enantiomers of underivatized amino acids on an RP_{18} column treated with Spi(τ-Dec). The mobile phase was 10^{-2} M acetate buffer, pH 5, containing 2×10^{-2} M copper acetate, at $T = 30\,°C$ and a flow rate of $1\,mL\,min^{-1}$; the detection wavelength was 230 nm.

nation) [27] whereas the overall hydrophobicity of the molecule determines sample retention. Enantioselectivity seems mostly dependent on the nature of the second residue.

Conclusions

The chiral stationary phase employed in this study is inexpensive, quick to prepare, and has good enantioselectivity toward underivatized amino acids and dipeptides. Such properties make it competitive with the commercial (and rather expensive) stationary phases with the same chiral recognition mechanism (ligand exchange).

The dynamic column-coating method has many specific advantages:

- it enables the preparation of new stationary phases by means of an easy procedure, with only minor limitations of column stability and the mobile phases which can be employed;
- it enables modulation of the performance of the chiral stationary phase – enantioselectivity (by careful modification of the structure of the chiral selector), retention (by varying the amount of chiral selector covering the support), and order of elution (by changing the chirality of the chiral selector);
- the stationary phase can be renewed frequently;
- some chiral selector optical impurity can be tolerated and consumption of the chiral selector is low (as for chiral stationary phase methods); and
- the chromatographic column employed is not chiral, is thus of wide commercial use and of relatively low cost (as for chiral mobile phase methods).

A chromatogram showing the simultaneous separation of five pairs of enantiomeric amino acids is presented in Figure 5.

Acknowledgements

This research was supported financially by the Ministry of University and Scientific and Technological Research (MURST, COFIN '98) and by the Italian National Research Council (CNR-990561403).

References

[1] Allenmark, S.G. *Chromatographic Enantioseparation: Methods and Applications*, Ellis Horwood, Chichester, **1988**.
[2] Krstulovic, A.M. Ed., *Chiral Separations by HPLC: Applications to Pharmaceutical Compounds* Ellis Horwood, Chichester, **1989**.
[3] Ahuia, S. Ed., *Chiral Separations by Liquid Chromatography* ACS Symposium Series 471, American Chemical Society, Washington, DC, **1991**.
[4] Subramanian, G. Ed., *A Practical Approach to Chiral Separations by Liquid Chromatography* VCH, Weinheim, **1994**.
[5] Beesley, T.E.; Scott, R.P.W. *Chiral Chromatography* John Wiley and Sons, Chichester, **1998**.
[6] Davankov, V.A.; Navratil, J.D.; Walton, H.F. *Ligand Exchange Chromatography* CRC Press, Boca Raton, Florida, **1988**.
[7] Rogozhin, S.V.; Davankov, V.A. *Chem. Commun.* **1971**, 490.
[8] Lepage, J.; Lindner, W.; Davies, G.; Seitz, D.; Karger, B.L. *Anal. Chem.* **1979**, *51*, 433–435.
[9] Lindner, W.; Lepage, J.; Davies, G.; Seitz, D.; Karger, B.L. *J. Chromatogr.* **1979**, *185*, 323–344.
[10] Tapuhi, Y.; Miller, N.; Karger, B.L. *J. Chromatogr.* **1981**, *205*, 325–337.
[11] Remelli, M.; Fornasari, P.; Pulidori, F. *J. Chromatogr. A* **1997**, *761*, 79–89, and references cited therein.
[12] Wachsmann, M.; Bruckner, H. *Chromatographia* **1998**, *47*, 637–642.
[13] Wan, Q.H.; Shaw, P.N.; Davies, M.C.; Barrett, D.A. *J. Chromatogr.* **1997**, *786*, 249–257.
[14] Miyazawa, T.; Minowa, H.; Imagawa, K.; Yamada, T. *Anal. Lett.* **1997**, *30*, 867–882.
[15] Yamazaki, S.; Saito, K.; Tanimura, T. *J. High Resol. Chromatogr.* **1998**, *21*, 561–564.
[16] Chilmonczyk, Z.; Ksycinska, H.; Cybulski, J.; Rydzewski, M.; Les, A. *Chirality* **1998**, *10*, 821–830.
[17] Chen, Z.L.; Lin, J.M.; Uchiyama, K.; Hobo, T. *Anal. Sci.* **2000**, *16*, 131–137.
[18] Remelli, M.; Piazza, R.; Pulidori, F. *Chromatographia* **1991**, *32*, 278–284.
[19] Remelli, M.; Fornasari, P.; Dondi, F.; Pulidori, F. *Chromatographia* **1993**, *37*, 23–30.
[20] Borghesani, G.; Pulidori, F.; Remelli, M.; Purrello, R.; Rizzarelli, E. *J. Chem. Soc., Dalton Trans.* **1990**, 2095–2100.
[21] Remelli, M.; Rossi, S.; Guerrini, R.; Pulidori, F. *Ann. Chim. (Rome)* **1995**, *85*, 503–518.
[22] Remelli, M.; Pulidori, F.; Guerrini, R.; Bertolasi, V. *J. Chem. Crystallogr.* **1997**, *27*, 507–513.
[23] Remelli, M.; Faccini, S.; Conato, C. *J. Chromatogr.* submitted.
[24] Rizzi, A.M. *J. Chromatogr.* **1991**, *542*, 221–237.
[25] Davankov, V.A. *J. Chromatogr. A* **1994**, *666*, 55–76.
[26] Arena, G.; Conato, C.; Contino, A.; Pulidori, F.; Purrello, R.; Remelli, M.; Tabbi, G. *Ann. Chim. (Rome)* **1998**, *88*, 1–12.
[27] Remelli, M.; Conato, C.; Agarossi, A.; Pulidori, F.; Mlynarz, P.; Kozlowski, H. *Polyhedron* **2000**, *19*, 2409–2419.

Received: Jul 16, 2001
Revised manuscript received: Sep 24, 2001
Accepted: Oct 23, 2001