Study of retention, efficiency and selectivity in chiral ligand-exchange chromatography with a dynamically coated stationary phase

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Abstract

A Bakerbond ODS column was dynamically coated with the chiral selector N^{τ}-n-decyl-L-histidine, and then loaded with copper(II) ions. A number of racemic mixtures of underivatized amino acids were resolved on such a column via chiral ligand-exchange chromatography. The most important experimental parameters influencing column efficiency, retention and selectivity (eluent flow-rate, analyte concentration, temperature and mobile phase composition) were extensively investigated. Results are discussed in light of CLEC theory and thermodynamic data on model systems in aqueous solution. The most likely structures for the stationary ternary complex are suggested.

Keywords: Retention; Column efficiency; Selectivity; Enantiomer separation; Amino acids; N^{τ}-n-Decyl-L-histidine

1. Introduction

A great number of chemicals of interest in different fields (pharmacology, biology, agriculture, synthesis, etc.) are optically active. In many cases it has been found that the two enantiomers have different activities vs. living organisms: thus, their optical purity must be kept under control, for both analytical and preparative purposes.

Many of the most efficient chiral separation methods are based on chromatography [1–7]. Particular interest is focused on amino acids, and various chiral separation methods have been reported. These can generally be classified as: those using (1) chiral stationary phases (CSP); (2) addition

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of a chiral reagent to the mobile phase (chiral mobile phase, CMP) and (3) precolumn derivatization of the analytes to form diastereomeric species which can be resolved on conventional columns.

Good success has been achieved in underivatized amino acid enantiomer separation via chiral ligand-exchange chromatography (CLEC) [7]. This technique was first introduced by Rogozhin and Davankov using a CSP derived from L-proline [8]. Metal complexation for enantiomer resolution by a chiral mobile phase additive was instead pioneered by Karger et al. [9–11]. 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, while the second is one of the components of the sample mixture. A suitable concentration of the complexing metal ion is present

both in the mobile and in the stationary phase. Thus, enantiomeric sample ligands can form diastereomeric complexes with the metal ion and the chiral selector: even small differences in stability can lead to enantiomer separation. To date, various chiral selectors have been used in CLEC, both as mobile phase or stationary phase chiral modifiers. They are often composed of an appropriate L-amino acid derivative [12–27].

In a previous work [20], a histidine-based chiral selector was synthesized so as to fully preserve the histidine complexing properties. L-Histidine was selectively alkylated with an n-decyl hydrocarbonic chain at the N^τ nitrogen atom in its imidazolic ring; this enabled it to adsorb strongly on a hydrophobic stationary phase for reversed-phase chromatography, thus forming a new chiral medium. The chiral selector N^τ-n-decyl-L-histidine, His(τ-dec), is shown in Fig. 1. Some other histidine derivatives have been employed in CLEC; these were either physically adsorbed [28] or chemically bonded [29–31] to the stationary phase in order to act as chiral selectors. However, in those cases, L-histidine was modified by reaction at its α-carboxy or α-amino group, which are known to act as donor groups when complexing the Cu(II) ion [32,33].

Recent work [20], based on the chiral modification of TLC plates with His(τ-dec), showed that this chiral selector possesses good resolution capabilities towards racemic mixtures of underivatized aromatic, amino-acid enantiomers. The dynamic coating technique was then employed to coat an HPLC–ODS packing with His(τ-dec) [24]. The interaction of this chiral selector with the hydrophobic support has been thoroughly investigated and the best experimental procedure to form the chiral layer has been found.

In the present work, the most important experimental parameters influencing efficiency, retention and selectivity of HPLC–ODS columns coated with the chiral selector His(τ-dec) are extensively studied, in underivatized amino acid enantiomer

resolution. The parameters considered are eluent flow-rate, analyte concentration, temperature and mobile phase composition (copper ion concentration, pH, organic modifier type and percentage, copper salt counter-ion, buffer concentration). Results are discussed in light of CLEC theory [34–36] and thermodynamic data on model systems in aqueous solution [32,33]. The most likely structures for the stationary ternary complex are suggested and the chiral discrimination mechanism is discussed.

2. Experimental

2.1. Materials

HPLC grade methanol (MeOH), ethanol (EtOH) and acetonitrile (ACN) and tetradistilled water were used as solvents. Copper(II) salts and acetic acid used in mobile phase preparation (Aldrich, Carlo Erba) as well as amino acids (Aldrich and Serva) were high purity products. L- and D-/L-spinacine (4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid, Spi) have been synthesized following the procedure described elsewhere [37]. Eluent pH was adjusted by addition of suitable amounts of a standard KOH solution, under potentiometric control. Synthesis and characterization of the chiral selector His(τ-dec) are described elsewhere [20].

2.2. Chromatographic equipment

HPLC measurements were made 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 990 photodiode-array detector (8-μl cell), coupled with a NEC APC III personal computer driven by Waters software. The wavelength range was 200–270 nm. By means of the spectrum analysis of the individual peaks, it was possible to distinguish the peaks of enantiomers (having the same spectrum) from system peaks, always present in the chromatogram.

A commercial Bakerbond (Baker, USA) stainless steel, analytical column (250×4.6 mm) for RP-HPLC was employed. Its packing consists of end-capped octadecyl silica (ODS): 5 μm spherical particles; surface area 170 m^2 g^{-1}; mean pore
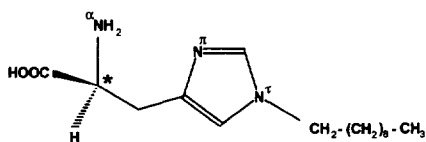

Fig. 1. Structure of the chiral selector N^τ-n-decyl-L-histidine, His(τ-dec).

diameter 12 nm; packing weight 3.8 g; typical loading 12% C (2 alkyl chains nm^{-2}).

2.3. Chiral column preparation

The His(τ-dec) solution was run through the column, at 0.2 ml min^{-1}, in open cycle, until the detector response proved stable. Chiral selector load was calculated from the His(τ-dec) breakthrough time, graphically measured and corrected for column dead volume. The modified column, after washing with water and with acetate buffers at increasing pH, [24] was then treated with the copper(II) acetate solution used as mobile phase.

2.4. Chromatographic measurements

Potassium nitrate has been used as column void volume marker (on the unmodified column). The capacity ratio (k') values are computed by taking the retention time (t_R) at the peak maximum. Column efficiency is calculated as reduced plate height (h) from the equation:

$$h = 0.18L(w_{1/2}/t_R)^2/d_p$$

where $w_{1/2}$ is the peak width measured at half peak height, d_p is stationary phase particle diameter and L the column length. Enantioselectivity, α, is always computed as k'_D/k'_L, where the subscript refers to the enantiomer; the elution order within a racemic sample was checked via the injection of single enantiomers. Peak asymmetry factor, A_s, is the ratio between the right half width at 1/10 of peak height and the corresponding left half width. Resolution (R_s) is the ratio between the peak distance, measured at the peak maximum, and the mean width, at the baseline, of the two considered peaks.

3. Results and discussion

The most important experimental parameters influencing both retention and resolution of the enantiomeric samples have been investigated on a number of chiral columns prepared as described above (Section 2.3). Each chiral column proved stable (i.e., with no significant change in retention and selectivity) after passing about 20 l of aqueous

eluent, i.e., after more than 500 analyses. The column life was, instead, markedly shorter when the eluent contained an organic modifier. Soaking, testing and covering the ODS column with new chiral selector required about three days. More than twenty racemic mixtures of amino acids have been used as enantiomeric samples; only in a few cases did it prove impossible to achieve good resolution.

3.1. Flow-rate

In Fig. 2 the column efficiency, calculated for D/L-Ala and D/L-Val, vs. the eluent flow-rate is shown. Plate height decreases markedly and linearly when reducing the mobile phase velocity, and at 0.2 ml/min a minimum has yet to be reached. As already observed, [35,36] this trend supports the hypothesis that the relatively low efficiency values usually found in CLEC depend mainly on slow exchange kinetics.

The HETP curve slope seems to depend on sample retention: generally the most retained samples show a better efficiency, as shown in Fig. 2. This behaviour is followed by the majority of the samples investigated (see also the efficiency data reported in Table 1 and Table 3 below), and is the opposite of what was reported by Rizzi [35] for chiral stationary phases based on Pro derivatives. Most likely, it is not only a coincidence that, in the present system, Pro and hydroxy-Pro (HyPro) constitute one of the few exceptions where the most retained enantiomer shows the highest plate height.

It is worth noting that the chromatographic theory [38] predicts, for systems with slow exchange kinet-
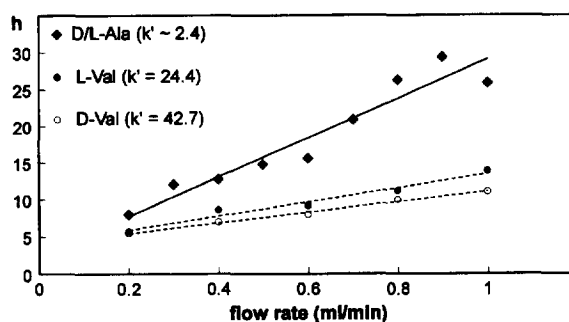

Fig. 2. Influence of flow-rate on plate height. Eluent: copper (II) acetate 0.1 mM in aqueous acetate buffer 2.0 mM, pH 5. His(τ-dec) loading: 0.20 mmol. Room temperature.

Table 1
Effect of the Cu(II) ion concentration in the mobile phase on retention (k'), selectivity (α), efficiency (h) and peak asymmetry (A_s)

Cu(II) concentration	0.1 mM				0.2 mM				0.5 mM		1.0 mM	
	k'	α	h	A_s	k'	α	h	A_s	k'	α	k'	α
L-Ala	11.8		–	1.4	8.8		–	–	6.2		4.8	
D-Ala	12.7	1.08	80.3	1.7	9.5	1.08	69.5	1.4	6.2	1.00	4.8	1.00
L-Glu	42.2		115.6	2.1	38.5		–	1.8	–		22.9	
D-Glu	48.4	1.15	–	–	44.2	1.15	–	–	–	–	24.9	1.09
L-Met	–		–	–	75.4		78.1	1.1	–		31.4	
D-Met	–	–	–	–	100.4	1.33	77.8	1.0	–	–	40.2	1.28
L-Orn	20.9		–	–	12.2		137.2	2.0	6.4		4.6	
D-Orn	19.0	0.91	270.9	2.8	11.1	0.91	156.1	2.3	6.2	0.96	4.4	0.96
L-Pro	27.9		105.2	2.3	20.3		73.6	2.3	13.6		10.7	
D-Pro	40.0	1.43	99.7	1.6	28.3	1.39	77.4	1.4	17.6	1.29	13.3	1.24
L-Thr	12.7		–	–	8.8		–	–	5.7		4.0	
D-Thr	12.7	1.00	–	–	8.8	1.00	–	–	5.7	1.00	4.0	1.00
L-Val	58.8		85.3	1.8	41.6		67.8	1.4	–	–	18.9	
D-Val	92.6	1.57	77.2	2.6	64.2	1.54	66.1	1.1	–	–	26.7	1.42

Eluent: unbuffered Cu(II) acetate aqueous solution; flow-rate: 1 ml/min; His(τ-dec) loading: 0.22 mmol; room temperature.

ics, that the mass transfer term C of the Van Deemter equation, which is responsible for the slope of the efficiency curve at flow-rates beyond the minimum, is directly proportional to the factor $k'/(1+k')^2$, and inversely to the rate constant k_d of the desorption step. While the first factor reaches a maximum for $k'=1$ and then decreases when k' increases, the second is a characteristic of each sample, under given experimental conditions. Moreover, the rate constant k_d is not necessarily correlated to the corresponding stability constant of the sorption complex (which rules the retention) even though Davankov observed that "there is no thermodynamic enantioselectivity without kinetic enantioselectivity" [36].

Finally, the enantioselectivity α remained unchanged varying the flow-rate, as expected, while the resolution increased with efficiency. Also the asymmetry (about 1.2–1.4 for Ala and 0.9 for Val) appeared unaffected by mobile phase velocity.

3.2. Copper(II) concentration

An increase in Cu(II) ion concentration in the eluent caused a continuous decrease in k' values, as shown in Table 1. In fact, the copper concentration controls "solvent strength" in that it affects the ligand-exchange mechanism of retention: an increase

in the Cu(II) ion content in the eluent shifts the equilibrium between fixed and mobile complexes in favour of the mobile phase. Above a Cu(II) ion concentration of 0.2 mM, one observes a relevant levelling effect, with negative consequences on column selectivity. On the other hand, increasing the copper content in the eluent from 0.1 to 0.2 mM, results in a small loss in selectivity while analysis time is shortened and column efficiency and resolution are improved.

3.3. Type and amount of organic modifier

Fig. 3 shows that the presence of an organic solvent in the eluent, even in low percentages, leads to a significant decrease in sample retention and to a dramatic loss in column selectivity. In fact, the organic modifier strongly affects all the hydrophobic interactions, and, in particular, those between the samples and the ODS support. Such interactions are the main sources both of retention and enantioselectivity for hydrophobic amino acids (see Section 3.9 below). The use of a low percentage of organic solvent can be useful to drastically reduce the run time for the most retained samples. However, this parameter should be used with special care because the column stability could be compromised.
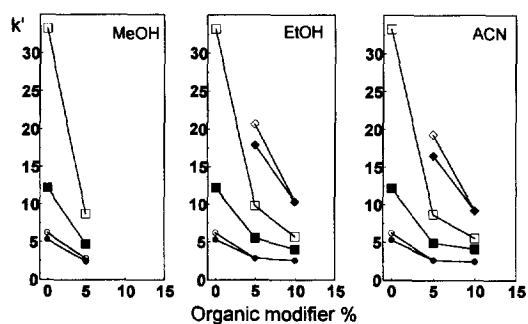
Fig. 3. Effect of organic modifier type and percentage in the mobile phase on retention and selectivity. Eluent: unbuffered copper (II) acetate 0.1 mM/organic modifier; flow-rate: 1 ml/min; His(τ-dec) loading: 0.25 mmol. Room temperature. Samples: (●) L-Ala; (○) D-Ala; (■) L-Pro; (□) D-Pro; (♦) L-Val; (◊) D-Val.

3.4. Eluent pH

Data reported in Table 2 shows that eluent pH is one of the most important parameters governing the retention of sample amino acids. All the samples become more and more retained as pH increases; the most important changes concern the amino acids bearing a hydrophobic side chain, while for the amino acids with a hydrophillic side chain, the effect seems less marked. Thermodynamic studies on aqueous solutions containing Cu(II) ions and two amino acids demonstrated that the formation of mixed complexes is favoured at pH values around the neutrality [39]. As a consequence, increasing the eluent pH should enhance the formation of the ternary stationary complexes; this accounts for the observed general increase in retention.

The eluent pH seems to have only a moderate effect on enantioselectivity: the α value generally reaches its maximum at a pH value between 4.5 and 5, where, in aqueous solution, the ternary complexes begin to prevail. An example of resolution of a number of racemic amino acids in a single run is shown in Fig. 4.

The dependence of column efficiency on eluent pH is shown in Table 3: h increases with pH for the least retained samples while the opposite trend is shown by the most retained amino acids (Met and n-Leu). For the amino acids with an aliphatic side chain (and also for Met) the plate height decreases when k' increases, even within each enantiomeric couple; the acidic amino acids (Asp and Glu) both

show the same h value when the pH is higher than 4, even though the respective k' values can be very different. Hypro shows peculiar behaviour in that the h value of the most retained D-enantiomer is always the highest; at pH 5.5 the peak of D-Hypro is notably flatter and broader than that of the L-enantiomer, although they are rather close.

Peak resolution reflects the changes in selectivity and efficiency of each sample: however, in general the first of these two parameters mainly determines the trend in R_s vs. eluent pH. An exception is due, in this case, to Met for which α assumes its best value at the lowest pH while R_s at the highest.

Finally, the peak asymmetry factor is generally closer to 1 at pH 5, with low fluctuations at different pH values. All the peaks are rather symmetric when the injected amount is reasonably low.

3.5. Column temperature

Table 4 shows that raising the column temperature from 25°C to 45°C reduces peak retention for all the samples, except for amino acids bearing an additional basic side group (Dabu, Orn, Lys, His, Arg) where it increases slightly. Enantioselectivity is invariably reduced by an increase in temperature, although no dramatic changes can be observed, in the explored range. Column efficiency is always increased by a higher temperature, due to the faster exchange kinetics, while resolution is, once again, most influenced by the corresponding α value. Column stability is not significantly affected by the temperature increase, within the explored range.

3.6. Cu(II) salt

Data reported in Table 5 shows that the anion of the copper salt employed in the eluent has little influence on either retention or enantioselectivity. Higher k' and α values have been found using copper acetate; however, these results can be ascribed to the higher pH value resulting from hydrolysis of the acetate, since the eluent was unbuffered. Also column efficiency remains practically the same with all the salts, while peak asymmetry seems to have a definite trend: $SO_4^{2-} > CH_3COO^- > NO_3^- > ClO_4^- > Cl^-$.

Table 2
Effect of eluent pH on peak retention (k'), enantiomer selectivity (α) and resolution (R_s)

	pH 3.8			pH 4.5			pH 5.0			pH 5.5		
	k'	α	R_s	k'	α	R_s	k'	α	R_s	k'	α	R_s
L-Ala	1.09			2.04			3.77			5.52		
D-Ala	1.20	1.11	0.8	2.32	1.14	1.0	4.34	1.15	1.1	6.32	1.15	0.8
L-Arg	0.15			0.55			1.54			2.94		
D-Arg	0.15	1.00	–	0.55	1.00	–	1.54	1.00	–	2.94	1.00	–
L-Asn	2.00			3.35			4.26			4.49		
D-Asn	2.00	1.00	–	3.35	1.00	–	4.26	1.00	–	4.49	1.00	–
L-Asp	20.38			23.34			24.41			25.10		
D-Asp	22.31	1.09	1.1	25.35	1.09	1.0	26.70	1.09	1.1	27.48	1.09	1.1
L-Dabu	0.09			0.30			0.69			1.03		
D-Dabu	0.09	1.00	–	0.24	0.80	0.6	0.54	0.78	0.8	0.81	0.79	0.7
L-Gln	1.87			3.65			5.96			7.68		
D-Gln	2.10	1.13	0.9	4.18	1.14	1.1	6.79	1.14	1.1	8.70	1.13	0.9
L-Glu	13.33			21.76			33.43			38.84		
D-Glu	15.48	1.16	1.3	25.47	1.17	2.0	38.74	1.16	1.8	44.79	1.15	1.7
L-His	0.52			1.45			2.02			2.31		
D-His	0.52	1.00	–	1.45	1.00	–	2.02	1.00	–	2.31	1.00	–
L-Leu	19.70			44.60								
D-Leu	29.04	1.47	4.9	66.04	1.48	4.9						
L-Lys	0.05			0.23			0.68			1.21		
D-Lys	0.05	1.00	–	0.23	1.00	–	0.68	1.00	–	1.21	1.00	–
L-Met	21.35			43.60			69.15					
D-Met	30.59	1.43	3.4	61.82	1.42	3.8	97.63	1.41	4.4			
L-n-Leu	24.27			55.87								
D-n-Leu	38.19	1.57	4.6	89.75	1.61	6.8						
L-n-Val				14.66			29.22			39.82		
D-n-Val				22.51	1.54	4.3	44.79	1.53	4.7	59.75	1.50	4.0
L-Orn	0.05			0.20			0.68			1.12		
D-Orn	0.05	1.00	–	0.18	0.89	0.6	0.49	0.72	0.8	0.82	0.74	0.8
L-Hypro	1.65			2.83			3.98			4.47		
D-Hypro	2.05	1.24	1.2	3.60	1.27	1.4	5.04	1.27	1.5	5.76	1.29	1.3
L-Pro	2.09			4.60			10.07			16.17		
D-Pro	3.12	1.49	2.2	8.10	1.76	3.0	14.99	1.49	3.3	23.41	1.45	3.1
L-Ser	1.48			2.55			3.99			4.44		
D-Ser	1.48	1.00	–	2.55	1.00	–	3.99	1.00	–	4.44	1.00	–
L-Spi	0.43			1.96			4.68					
D-Spi	0.82	1.91	2.1	3.89	1.99	3.0	6.47	1.38	–			
L-Thr	2.76			4.62			6.27			7.05		
D-Thr	2.76	1.00	–	4.62	1.00	–	6.27	1.00	–	7.05	1.00	–
L-Val				15.35			29.59			40.21		
D-Val				25.97	1.69	5.4	49.37	1.67	5.2	65.90	1.64	4.4

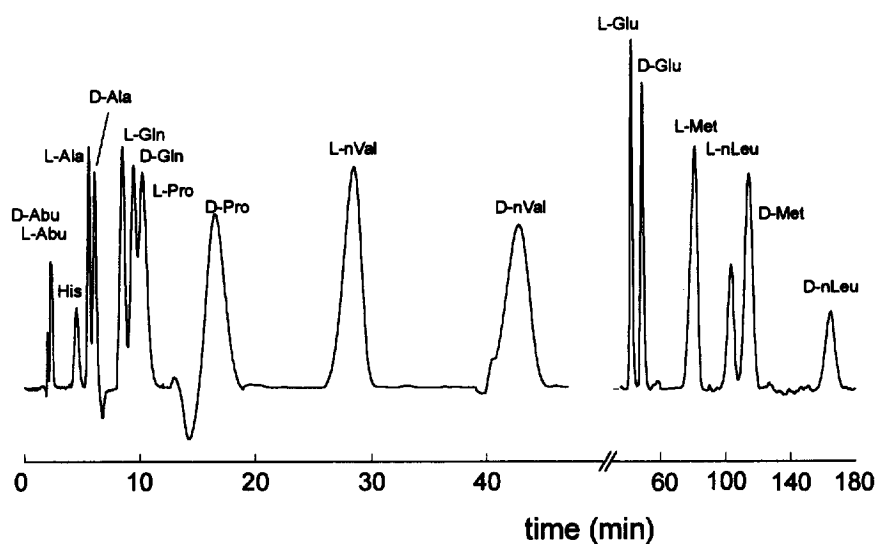Eluent: Cu(II) acetate 0.2 mM, acetate buffer 2.0 mM; flow-rate: 1 ml/min; His(τ-dec) loading: 0.20 mmol; room temperature.

Fig. 4. Resolution of some racemic amino acids on a Bakerbond C_{18} column (250×4.6 mm, 5 μm), coated with His(τ-dec) (0.20 mmol). Eluent: Cu(II) acetate 0.2 mM, acetate buffer 2.0 mM, pH 4.5; flow-rate: 1 ml/min; room temperature.

Table 3
Effect of eluent pH on column efficiency (h) and peak asymmetry (A_s)

	pH 3.8		pH 4.5		pH 5.0		pH 5.5	
	h	A_s	h	A_s	h	A_s	h	A_s
L-Ala	16	1.1	24	1.2	36	1.0		
D-Ala	17	1.1	25	1.2	34			
L-Asp	18		19		20		21	
D-Asp	18		20		21		23	
L-Gln	24		31		35		53	
D-Gln	28		30		35			
L-Glu			18	1.5	20	1.6	23	1.6
D-Glu	25	0.8	17	1.6	20	1.7	22	1.6
L-Met	49		34	0.6	18	1.0		
D-Met	21		20	1.0	18	0.9		
L-n-Leu	42	0.5	16	0.8				
D-n-Leu	20	0.7	14	0.8				
L-n-Val			33	0.7	25	1.0	32	1.0
D-n-Val			25	0.7	24	0.9	28	1.2
L-Hypro	35	1.1		0.9	48	1.0	68	
D-Hypro	43		44	1.0	49	1.0	91	
L-Val			29	0.7	28	1.0	36	0.9
D-Val			23	0.8	26	1.0	35	0.8

Eluent: Cu(II) acetate 0.2 mM, acetate buffer 2.0 mM; flow-rate: 1 ml/min; His(τ-dec) loading: 0.20 mmol; room temperature.

Table 4
Effect of column temperature on peak retention and enantio-selectivity

	Column temperature (°C)					
	25		35		45	
	k'	α	k'	α	k'	α
D/L-Dabu	2.8	1.00	3.0	1.00	3.3	1.00
L-Glu	16.8		16.1		14.8	
D-Glu	19.3	1.15	18.3	1.14	16.7	1.12
D/L-His	3.5	1.00	3.6	1.00	3.7	1.00
L-Leu	46.3		43.6	–	40.5	–
D-Leu	64.3	1.39	60.3	1.38	55.3	1.37
L-Met	39.5		34.6		28.0	
D-Met	52.8	1.34	46.1	1.33	36.9	1.32
L-Pro	7.2		6.9		6.6	
D-Pro	10.8	1.49	9.6	1.38	9.0	1.35
L-Val	18.2		16.8		15.8	
D-Val	28.3	1.55	25.9	1.54	23.9	1.52

Eluent: Cu(II) acetate 0.5 mM, unbuffered; flow-rate: 1 ml/min; His(τ-dec) loading: 0.21 mmol.

Table 5
Effect of the Cu(II) counter ion on retention (k') and selectivity (α)

	Acetate		Sulfate		Nitrate		Perchlorate		Chloride	
	k'	α	k'	α	k'	α	k'	α	k'	α
L-Ala	6.5		5.9		5.5		4.9		4.8	
D-Ala	7.2	1.12	6.4	1.09	5.9	1.06	5.2	1.05	5.4	1.11
L-Glu	33.3		23.7		35.1		18.6		–	
D-Glu	38.8	1.17	26.0	1.09	39.3	1.12	20.7	1.11	–	–
L-Met	64.3		51.2		48.8		41.9		44.8	
D-Met	86.9	1.35	65.6	1.28	64.8	1.33	54.8	1.31	58.7	1.31
L-Orn	–		6.8		4.8		5.7		4.0	
D-Orn	–	–	5.9	0.86	4.4	0.91	5.2	0.91	3.6	0.91
L-Pro	14.6		14.7		13.3		12.6		12.4	
D-Pro	22.1	1.51	19.1	1.30	17.6	1.32	16.1	1.27	16.0	1.29
L-Val	32.3		28.5		26.9		22.7		23.8	
D-Val	52.8	1.63	41.2	1.45	40.8	1.52	33.4	1.47	35.6	1.49

Eluent: Cu(II) salt 0.5 mM, unbuffered; flow-rate: 1 ml/min; His(τ-dec) loading: 0.25 mmol; room temperature.

3.7. Acetate buffer concentration

The concentration of acetate buffer in the eluent, when both pH and copper concentration are kept constant, has interesting effects on retention and selectivity of amino acids, due to the simultaneous action of buffer index and ionic strength variation. Table 6 shows that, increasing the buffer concentration, retention decreases for most samples: rather quickly for Asp and Glu and more slowly for the others. Enantioselectivity follows the same trend. The opposite behaviour is followed, instead, by the basic amino-acids, for which an increase in buffer concentration leads to a significant increase in retention and to an improvement in selectivity. This result has the important consequence that, with the most concentrated buffers, it is possible to resolve the Arg and Lys enantiomers.

The effect of increasing the buffer concentration on column efficiency is not well defined: a general worsening seems to take place, especially in the case of most retained samples (Val, Leu, Met). The same trend is followed by resolution as well.

3.8. Sample size and detection limit

Variable amounts (0.2–2 μg) of sample, generally dissolved in the mobile phase in use, were injected. Some detection limits have been computed: 30 ng for

L-Val [$\lambda = 240$ nm; eluent: Cu(II) acetate 0.2 mM, acetate buffer 2.0 mM, pH 4.8]; 7 ng for L-Spi [$\lambda = 240$ nm; eluent: Cu(II) acetate 0.2 mM, acetate buffer 2.0 mM, pH 3.8]. However, it is worth noting that the detection limit of each sample depends not only on its retention and on the employed wavelength but also on the Cu(II) ion concentration (which determines the baseline level) and on the eluent pH; these parameters both govern the formation equilibria of Cu(II) complexes in the mobile phase.

3.9. Chiral recognition mechanism

It is well known that His is a potentially tridentate ligand which can bind the Cu(II) ion in two fundamental ways: histamine-like (*hm-like*) (i.e., by means of its amino and π-imidazole nitrogen atoms) and glycine-like (*gly-like*) (i.e., by means of its carboxylic and amino groups). The latter is preferred at the most acidic pH values, where protonated (binary or ternary) complexes are formed; in the protonated ternary complexes, the second amino acid is bound in *trans* position on the equatorial plane of the complex. On the other hand, the *hm-like* coordination mode prevails when pH is raised above 4.5, approximately. Here, the mixed complexes become unprotonated and the amino groups of the two ligands are prevalently bound in the *cis* position,

Table 6
Effect of acetate buffer concentration in the mobile phase on peak retention and enantiomer selectivity

	Buffer concentration							
	0.001 M		0.005 M		0.020 M		0.100 M	
	k'	α	k'	α	k'	α	k'	α
L-Ala	3.9		3.6		3.1		2.2	
D-Ala	4.3	1.11	4.0	1.10	3.4	1.10	2.4	1.07
L-Arg	–		–		4.2		4.7	
D-Arg	–		–		3.7	0.87	4.1	0.87
L-Dabu	–		1.5		2.1		2.6	
D-Dabu	–	–	1.5	1.00	1.7	0.81	2.2	0.84
L-Gln	–		–		4.7		3.6	
D-Gln	–	–	–	–	5.1	1.07	3.6	1.00
L-Glu	29.0		18.0		10.4		4.1	
D-Glu	34.0	1.17	20.9	1.16	11.9	1.14	4.6	1.13
L-Leu	55.5		51.4		44.4		28.8	
D-Leu	79.8	1.44	72.5	1.41	60.8	1.37	38.5	1.34
L-Lys	–		1.6		2.1		2.5	
D-Lys	–	–	1.4	0.86	1.8	0.86	2.2	0.87
L-Met	43.8		43.0		38.4		26.4	
D-Met	59.8	1.37	58.0	1.35	50.7	1.32	34.2	1.30
L-Orn	1.1		1.5		1.9		2.4	
D-Orn	0.8	0.79	1.2	0.77	1.5	0.78	2.0	0.82
L-Pro	7.4		7.1		5.9		3.9	
D-Pro	12.4	1.69	10.8	1.53	8.8	1.50	5.3	1.37
L-Val	20.4		19.1		16.5		10.4	
D-Val	33.6	1.64	30.5	1.60	25.5	1.54	15.9	1.53

Eluent: Cu(II) acetate 0.5 mM, acetate buffer pH 5; flow-rate: 1 ml/min; His(τ-dec) loading: 0.21 mmol; room temperature.

again in the equatorial plane. This behaviour is well assessed for ternary Cu/His complexes in batch aqueous solution [32,33,40] and supported by crystallographic data [41–44].

According to the above considerations, the two most likely structures of a mixed stationary complex (*hm-like* and *gly-like*) with an L-(*homo*-chiral complex) or a D-amino acid (*hetero*-chiral complex) are shown in Fig. 5. It is worth noting that the unprotonated chiral selector can always bind the Cu(II) ion in a tridentate fashion.

These suggested structures are in good agreement to those already proposed by Davankov for the CLEC system based on the use of N^{α}-decyl-L-histidine as a chiral selector [28]. However, the different position of the alkyl chain causes a very different orientation of the sorption complex with respect to the hydrophobic support, with important changes in column retention and selectivity, especially as far as the hydrophilic amino acids are concerned. In fact, in that CLEC system, Lys, Arg and Glu were not resolved at all, while Thr and Ser enantiomers were well separated; Asp and Dabu had the opposite selectivity with respect to the present chromatographic system.

In both the structures shown in Fig. 5, the side group of the amino acid sample is closer to the hydrophobic surface in the case of the D-enantiomer; when this group is hydrophobic, an attractive interaction with the C_{18} chains can take place, increasing the retention of this enantiomer. The possibility of a *cis-trans* equilibrium certainly reduces the column selectivity, as already observed by Davankov [28], with respect to other CLEC systems in which the
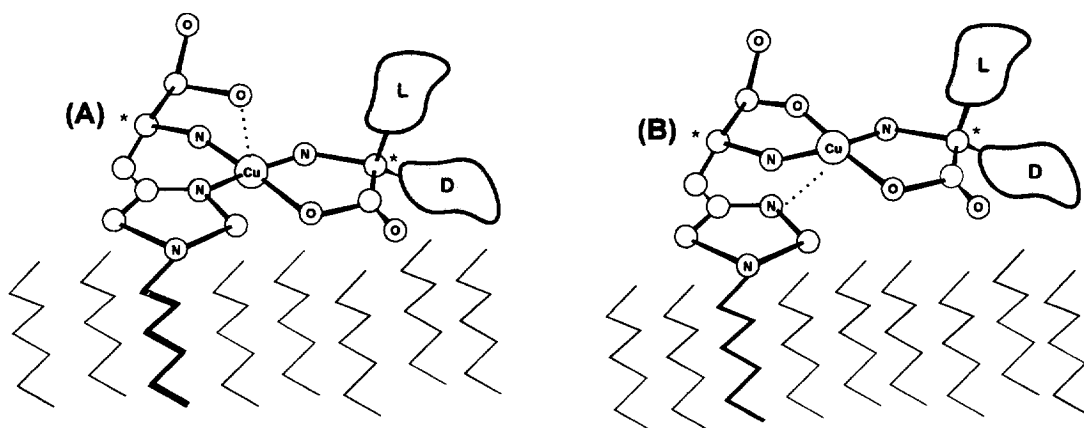
Fig. 5. Structural hypotheses for the stationary Cu(II) complex of the chiral selector His(τ-dec) with an amino acid sample. The position of the side chain of the sample enantiomer is indicated by the letters L or D. His(τ-dec) coordination mode: (A) *hm-like*; (B) *gly-like*.

coordination mode is unique. On the other hand, this effect is most likely responsible for the relatively high retention obtained for both the enantiomers of hydrophobic amino acids.

Pro and Met (respectively having an aliphatic side ring and a thio-ether side chain) behave like hydrophobic amino acids: rather high retention, good enantiomer resolution with the L-enantiomer always eluted first.

Basic amino acids (Dabu, Arg, His, Lys, Orn), having an additional basic side group, mostly protonated in the explored pH range, show low retention and a reversed enantioselectivity (D- before L-). In this case, no additional hydrophobic interaction with the ODS support can be generated by the side chain. However, in the *homo*-chiral complex, an attractive electrostatic interaction is possible between the positively charged ω-group and the negatively charged carboxy group of His(τ-dec), when the structure of the complex is that shown in Fig. 5A. This seems to be the source of the observed enantioselectivity. In this case the agreement with solution data on model systems [33] is pretty good. In fact, in aqueous solution containing Cu(II) ions L- or D-His and an L-basic amino acid, a significant thermodynamic stereoselectivity favours the formation of *homo*-chiral ternary complexes. This behaviour was attributed to the presence of an additional intramolecular interaction between the ligands, as described above.

The opposite behaviour is instead shown by the amino acids bearing an additional carboxy group

(Asp, Glu), which is certainly deprotonated (and negatively charged) in the explored pH range. In this case we can observe that, in the *hetero*-chiral complex, the carboxy group can interact with the protonated imidazole nitrogen atom of the chiral selector, when the structure is as in Fig. 5B. The same carboxy group can also bind the central metal ion in the axial position; this additional coordination is possible for both the enantiomers and it has greater effect on the relatively high retention of Asp and Glu than on enantioselectivity. The contribution to enantioselectivity due to structure (A) appears to be of minor importance but again in the same direction. In fact, the *homo*-chiral complex can be destabilized by the presence, on the same side of the complex, of the two carboxy groups not bound to the equatorial plane of the complex.

A direct comparison of the overall stability constants for ternary *homo*- and *hetero*-chiral Cu(II) complexes of His with amino acids, in aqueous solution, [32,33] and chromatographic retention and selectivity data, proved rather unsuccessful. This point has already been evidenced by Davankov [34] who concluded that "an immediate relationship... only exists in a single case, namely that with the chiral selector residing entirely in the mobile phase". The presence of the hydrophobic support plays a fundamental role in column retention and selectivity, especially when amino acids bearing a hydrophobic side chain are concerned. Nevertheless, it is worth underlining that the knowledge of the different

possible structures of model complexes and of the possible additional intramolecular interactions between the ligand side chains proved very useful in interpreting the chromatographic behaviour of the various samples. In addition, the same arguments can be employed to design new and more selective chiral selectors; this will be the subject of future work.

Acknowledgments

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