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Chiral separation of amino acids by copper(II) complexes of tetradentate diaminodiamido-type ligands added to the eluent in reversed-phase high-performance liquid chromatography: a ligand exchange mechanism

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Abstract

In this paper we report a study on the mechanism of the enantiomeric separation of unmodified D,L-amino acids in RP-HPLC by copper(II) complexes of two tetradentate diaminodiamido ligands, (*S*,*S*)-*N*,*N*′-bis(phenylalanyl)ethanediamine (PheNN-2) and (*S*,*S*)-*N*,*N*′-bis(methylphenylalanyl)ethanediamine (Me_2PheNN-2), added to the eluent. The aim is to investigate whether and how a copper(II) complex with no free equatorial positions can perform chiral discrimination of bidentate analytes such as unmodified amino acids. The problem is approached in a systematic way by: (a) varying the different chromatographic parameters (pH, selector concentration, eluent polarity); (b) performing chiral separation with the selector adsorbed on the stationary phase; (c) studying the ternary complex formation of these ligands with D- and L-amino acids in solution by glass electrode potentiometry and electrospray ionization MS. All the experimental data are consistent with a mechanism of chiral recognition, based on ligand exchange, which involves as selectors the species $[Cu_2L_2H_{-2}]^{2+}$ and $[CuLH_{-2}]$ and proceeds by displacement of two binding sites from the equatorial positions, giving rise to the ternary species $[CuLA]^{+}$ and $[CuLH_{-1}A]$. The most important factor responsible for chiral discrimination seems to be the affinity of the diastereomeric ternary complexes for the stationary phase since no enantioselectivity is observed in solution.

Keywords: Enantiomer separation; Stability constants; Ligand exchange; Amino acids; Copper(II) complexes; Tetradentate ligands

1. Introduction

During the past years a wide variety of molecular systems based on the principle of ligand exchange chromatography [1] were proposed for the enantiomeric separation of bidentate substrates in high-

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performance liquid chromatography (HPLC). It involves reversible formation of diastereomeric complexes of transition metal ions (copper(II) is the most common) with chiral ligands (generally amino acids or derivatives) and enantiomers. When the ligand is linked to a solid support (chiral stationary phases), it can form diastereomeric adsorbates with enantiomers of different stabilities, leading to the separation of the racemate [2–11]. When the chiral complex is added to the mobile phase (chiral eluent), the mechanism of chiral discrimination is, in general, quite complex, and it may involve a series of complexation equilibria in the mobile and in the stationary phase as well as partition equilibria of the different species between the two phases [12–18]. Chiral coated stationary phases have also been proposed, in which the selector is dynamically adsorbed on the column stationary phase [19–28].

As far as the mechanism is concerned, that ligand exchange does occur in the chromatographic system has been demonstrated by us some years ago by means of a copper(II) complex of a bidentate ligand, (*S*)-tryptophanamide, (Cu/L=1:2) added to the eluent [29,30]. Upon formation of the ternary complex with the amino acid, one ligand molecule is displaced from the initial binary complex, which is not fluorescent, giving rise to a specific fluorescent signal, corresponding to tryptophanamide. It was found that the chromatographic separation was not due to the stereoselectivity observed in solution, rather to the different affinity of the diastereomeric complexes for the stationary phase [31].

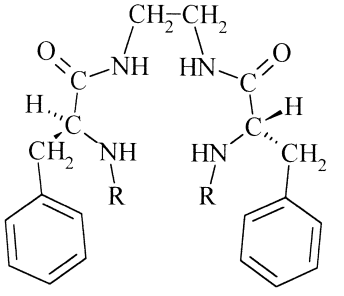
In contrast, with terdentate ligands the mechanism of chiral recognition was found to depend on the strength of the three binding sites: with ligands bearing two strong donor groups (an amino and an amido) and a weak binding site (an hydroxyl group), such as in N^2-[(*S*)-2-hydroxypropyl]-(*S*)-phenylalaninamide [32,33], the last one is easily displaced during diastereomeric complex formation. Instead, with ligands with three strong binding sites, such as the diaminoamido ligand *N*-(*S*)-phenylalanylethanediamine (PheN-2), the selector $[CuLH_{-1}]^+$ was shown to maintain the coordination of the three nitrogen atoms around Cu^{II} in the mixed complex $[CuLH_{-1}A]$, the bidentate enantiomer binding at the remaining equatorial position and eventually at the apical position. In the latter case chiral discrimination of unmodified and modified amino acids turned out to be less efficient than with bidentate ligands [34].

The formation equilibria in aqueous solution of the ternary copper(II) complexes of bidentate amino acid amides [31,35] and terdentate diaminoamido ligands [34] with D- or L-amino acids allowed us to clarify the thermodynamic aspects of the recognition mechanism. However, stereoselectivity in solution contributed but a minimum to the overall discrimination process and the major factor involved in the recognition mechanism appeared to be the affinity of the complexes for the stationary phase, mainly due to the lipophilicity of the selector.

The problem remains whether chiral discrimination still occurs via ligand exchange also with copper(II) complexes of tetradentate diaminodiamido ligands, which were shown to give very good enantiomeric separation of dansyl-amino acids [36,37].

In this paper we report the enantiomeric separation of unmodified amino acids by HPLC by means of copper(II) complexes of the tetradentate ligands (*S*,*S*)-*N*,*N*′-bis(phenylalanyl)ethanediamine (PheNN-2) (**1a**) and (*S*,*S*)-*N*,*N*′-bis(methylphenylalanyl) ethanediamine (Me_2PheNN-2) (**1b**) (Fig. 1) added to the eluent.

L-Phenylalanine was maintained as chiral building block for the ligands on account of its lipophilicity and for analogy with the corresponding bidentate amide and with the terdentate diaminoamido ligands,



1a R = H
1b R = CH_3

Fig. 1. Structures of the ligands PheNN-2 (**1a**) and Me_2PheNN-2 (**1b**).

which were previously used for the enantiomeric separation in HPLC [29,34]. *N*-Methylation of phenylalanine was performed mainly for practical reasons, i.e., to allow for the detection of unmodified amino acids by post-column derivatization with orthophthalaldehyde (OPA).

In order to evaluate the thermodynamics of complexation and to define the binary and ternary species potentially involved in the chiral discrimination of amino acids, we have investigated the solution equilibria of the Cu^{2+}–PheNN-2 (or Me_2PheNN-2)–D- or L-amino acid systems by glass electrode potentiometry and electrospray ionization mass spectrometry (ESI-MS).

Moreover, adsorption of the selector on the stationary phase was studied in order to evaluate the relative importance of complex formation in solution and on the stationary phase.

2. Experimental

2.1. Reagents

D,L-, D- and L-amino acids were obtained from Sigma (St. Louis, MO, USA); OPA, boric acid, EDTA were from Fluka (Buchs, Switzerland). Acetonitrile (LC-grade), methanol (LC grade), copper(II) acetate, sodium acetate were obtained from Carlo Erba (Milan, Italy); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA).

2.2. Synthesis of the ligands

The ligands PheNN-2·2HCl [37,38] and Me_2PheNN-2·2HCl [39] were synthesized as previously reported, purified and stored as crystalline dihydrochlorides.

2.3. Chromatographic analyses

Chromatographic analyses were performed with Waters Model 510 pumps equipped with a Model 470 fluorescence detector (λ_{ex}=330 nm and λ_{em}= 440 nm for OPA derivatives) (Waters, Milford, MA) or with a Model 759A absorbance detector (Applied Biosystems, Nieuwerkerk a/d Ijssel, NL) set at 254 nm. The system was controlled by a MAXIMA 820 Chromatography Workstation for data handling. C_{18} Spherisorb ODS-2 columns (3 μm, 15×0.46 cm) were used.

2.4. Preparation of the chiral mobile phase

The mobile phase was prepared by dissolving the ligand and copper(II) acetate (1:1 molar ratio, concentration range 0.05–2 m*M*) in bidistilled water or in a water–methanol or water–acetonitrile mixture. The pH was adjusted either with NaOH or CH_3COOH. The eluent was filtered and degassed under reduced pressure on HPLC filters (0.45 μm). In order to equilibrate the system, the mobile phase was allowed to flow through the column until constant elution times were obtained.

The column hold-up volume (V_0) was determined from the elution time of an unretained solute (acetic acid).

2.5. Preparation of the derivatizing agent: OPA

A solution of OPA (0.8 g) in a mercaptoethanol–methanol (1:1, v/v, 8 ml) mixture was added to 1 l of potassium borate buffer (0.3 *M*, pH 10), containing ethylenediaminetetraacetic acid (EDTA) (2.5 g), under vigorous magnetic stirring. The solution was filtered (0.45 μm filters) and degassed under reduced pressure.

2.6. Adsorption of the chiral selector onto the stationary phase

Adsorption of the selector onto the C_{18} column stationary phase was performed by eluting with a 0.5 m*M* solution of Me_2PheNN-2–Cu(II) (1:1) at pH 7.5. Saturation was controlled by comparing the absorbance at 503 nm (λ_{max} for the species $[CuLH_{-2}]$) of the eluate from the column. Quantitative recovery of the selector was obtained by column washing with MeOH.

2.7. Potentiometric measurements

The experiments were carried out at T= 25.0±0.1°C and I=0.1 *M* (KCl) under a moistened nitrogen stream. Computer-controlled titrations were

performed using a 5-ml Metrohm 655 Dosimat burette and a Crison MICROPH2002 digital voltmeter equipped with a Hamilton combined glass electrode (P/N: 238 000). The electrodic chain was calibrated in terms of $[H^+]$ by titrating HCl with standard KOH solution (hereafter, $pH=-\log [H^+]$). The PC program BEATRIX [40], based on the Gran method, was used to calculate the equivalence volume, V_e, the electrode couple standard potential, E^0, and pK_w [13.76 (1)]. Stock solutions of the amino acids (ca. 0.02 *M*) were prepared by weight and used within 3–4 days, whereas appropriate amounts of PheNN-2 and Me_2PheNN-2 dihydrochlorides were weighed for each titration. Cu^{II}, KOH and HCl solutions were prepared and standardized as already reported [31]. For each of the ternary systems examined, 3–5 alkalimetric titrations were performed with two different Cu/L/A ratios: 1:1:1 and 1:1.5:1.5. c_{Cu} was 1–2 m*M* for PheNN-2 and 1–1.5 m*M* for Me_2PheNN-2. The pH range explored was ca. 3.0–11.0 for both ligands, except for the Me_2PheNN-2–L-Trp system, where precipitation occurred at pH ca. 7.5.

The stability constants were calculated using the non-linear least-squares program HYPERQUAD [41]. Protonation and binary Cu^{II} complexation constants of the ligands were used as fixed parameters in the refinement of trial log β values of the ternary species. For each system, the data from different titrations were treated in a unique batch.

2.8. ESI-MS measurements

ESI-MS spectra were recorded on a ZMD mass spectrometer (Micromass, Manchester, UK) fitted with a pneumatically-assisted electrospray probe. Samples were analyzed by direct infusion at 10 μl/min. In the positive-ion mode the following conditions were used: ES capillary, 3.0 kV; cone, 30 V; extractor, 2 V; radio frequency (RF) lens, 0.1 V; source block temperature, 80°C; desolvation temperature, 150°C; cone and desolvation gas (N_2) flow-rates, 1.6 and 8 l/min, respectively. Mass spectra were scanned in the m/z range 50 to 1500 at 5.92 s/scan with an interscan delay of 180 ms. In the negative-ion mode the following conditions were used: ES capillary, 3.08 kV; cone, 22 V; extractor, 4 V; RF lens, 0.26 V; source block temperature, 80°C; desolvation temperature, 150°C; cone and desolvation gas (N_2) flow-rates, 1.6 and 8 l/min, respectively. Mass spectra were scanned in the m/z range 100 to 1500 at 2.96 s/scan with an interscan delay of 140 ms. Data were processed by using the spectrometer software (MassLynx NT version 3.4). Aqueous solutions of the ternary system Cu^{II}–Me_2PheNN-2–D-Trp (1:1.5:1.5) (c_{Cu}=1 m*M*) at pH 7.1 and 11.6 were examined.

3. Results and discussion

3.1. Enantiomeric separation of unmodified amino acids: selector added to the eluent

The ligands PheNN-2 (**1a**) and Me_2PheNN-2 (**1b**) (Fig. 1) were synthesized as previously reported [37–39].

Solutions of either PheNN-2 or Me_2PheNN-2 and Cu^{II} (1:1 molar ratio), at appropriate pH, were used as chiral eluents in reversed-phase HPLC for the enantiomeric separation of unmodified amino acids, by using a C_{18} column and a UV detector at 254 nm. With PheNN-2 it was quite difficult to obtain good chromatograms, owing to the high absorbance of the colored solution in the UV–Vis range and to the presence of different system peaks, commonly observed with a UV detector and eluents containing copper(II) complexes [42]. However, the enantiomeric separation of D,L-alanine (Ala) and D,L-proline (Pro) was obtained, as reported in Fig. 2. The enantioselectivity factors (α=1.32 for Ala and α=1.67 for Pro) were good, although the second eluting peak was quite broad.

To overcome the problem, the methylated ligand Me_2PheNN-2 was used, which allowed to perform post-column derivatization with OPA, forming highly fluorescent derivatives, detectable with a fluorescence detector (λ_{ex}=330 nm and λ_{em}=440 nm) at pmol levels. With this ligand very good enantiomeric separations were obtained both for polar and apolar amino acids (Fig. 3), with a different elution order for the polar (L<D) and for the apolar amino acids (D<L), as already observed for the analogous bidentate ligand *N*-methyl-(*S*)-phenylalaninamide (MePhe-NH_2) [29].

The chromatographic behavior was studied as a

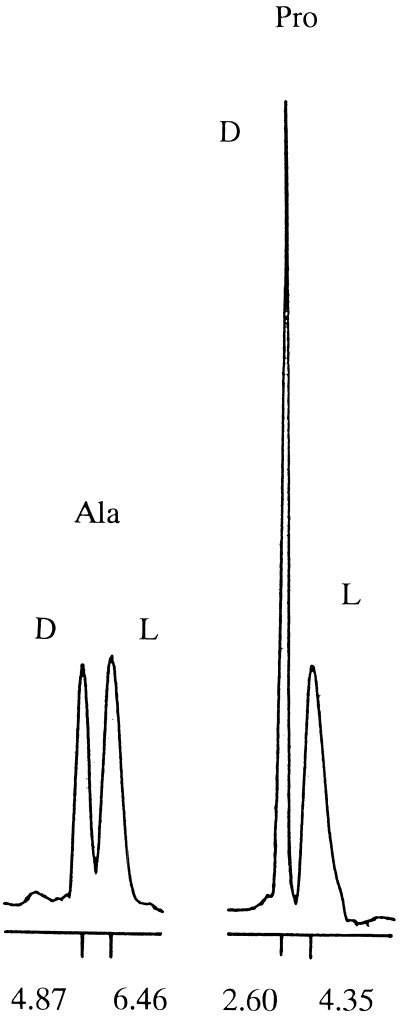


Fig. 2. Enantiomeric separation of D,L-amino acids with the system PheNN-2–Cu^{II}. Conditions: eluent, 0.5 m*M* PheNN-2 and 0.5 m*M* copper(II) acetate in water, pH 6.0; column, Spherisorb ODS2 (3 μm, 15×0.46 cm); room temperature; flow-rate, 1.0 ml/min; detection, UV (254 nm).

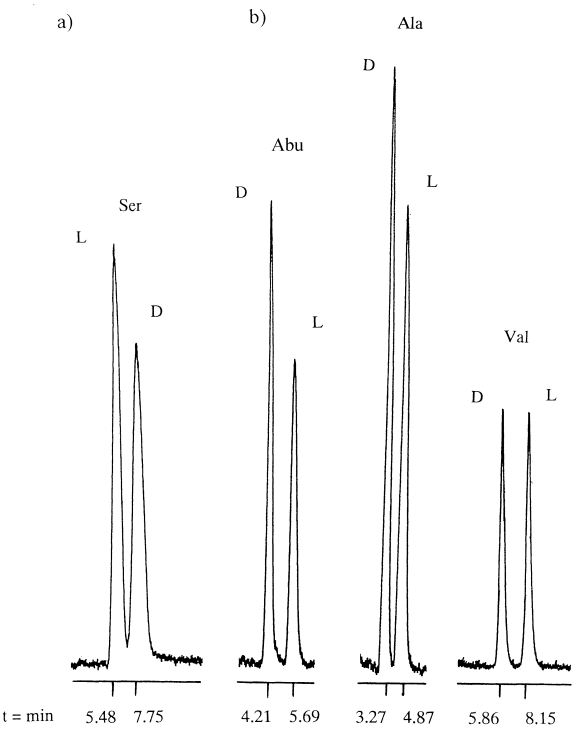


Fig. 3. Enantiomeric separation of D,L-amino acids with the system Me_2PheNN-2–Cu^{II}. Conditions: eluent, 0.5 m*M* Me_2PheNN-2 and 0.5 m*M* copper(II) acetate in water pH 6.0; column Spherisorb ODS2 (3 μm, 15×0.46 cm); flow-rate, 1.0 ml/min; fluorescence detection (post-column derivatization with OPA, $\lambda_{ex}=330$ nm, $\lambda_{em}=440$ nm); column temperature, 52°C (a) and 35°C (b).

function of pH in the range 5.5–7.5 and of the percentage of the organic modifier (MeOH). As the pH increased, both capacities (k) and separation factors (α) increased for all amino acids. Data for serine, glutamic acid and α-aminobutyric acid (Abu) are shown in Fig. 4a–c.

Acceptable retention times were obtained by adding MeOH to the aqueous solution, although a decrease in enantioselectivity was observed (e.g., for D,L-Ala: 10% MeOH: $k_L=6.61$, $k_D=4.54$, $\alpha=1.46$; 20% MeOH: $k_L=3.24$, $k_D=2.72$, $\alpha=1.19$).

The capacity and the enantioselectivity factors of D,L-Ala and D,L-Abu were studied as a function of the chiral selector concentration in the eluent (Fig. 5).

With both amino acids, enantiomers started to be separated at a selector concentration in the eluent higher than 0.025 m*M*. By increasing the concentration, the capacity factors increased for both enantiomers, but to a higher extent for the L-enantiomer. Enantioselectivity reached a maximum at a selector concentration of 0.5 m*M*, then decreased. This phenomenon is evidently linked to the adsorption of the selector on the stationary phase and strongly varies with the concentration of the selector in the mobile phase. At high concentration of the selector in the mobile phase, the latter predominantly

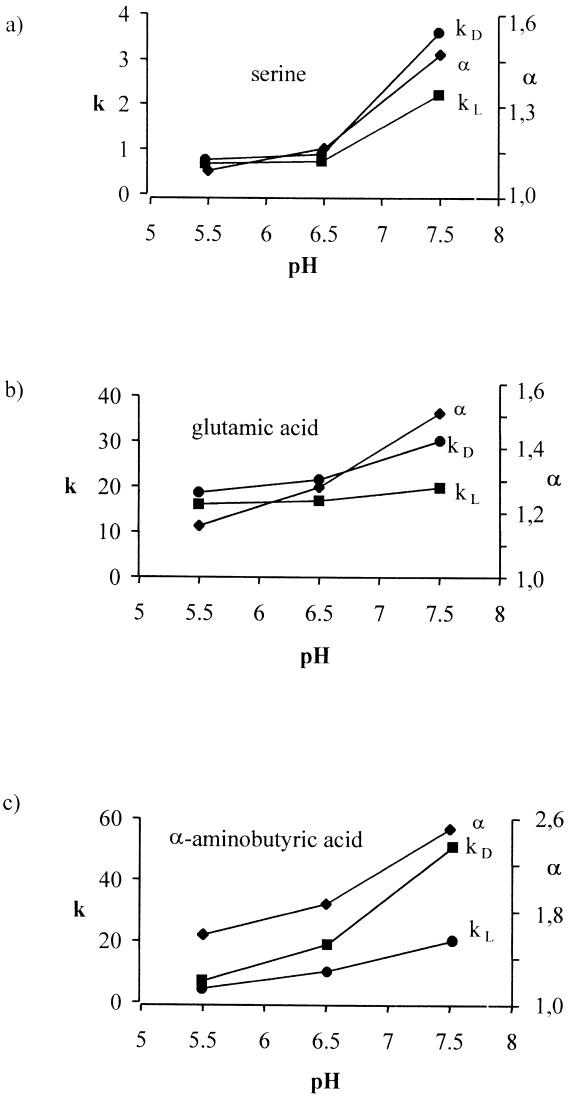


Fig. 4. Effect of pH on the capacity (k) and enantioselectivity factors (α). Conditions: eluent, 0.5 m*M* Me_2PheNN-2 and 0.5 m*M* copper(II) acetate in water; column, Spherisorb ODS2 (3 μm, 15×0.46 cm); room temperature; flow-rate, 1.0 ml/min; fluorescence detection (post-column derivatization with OPA, $\lambda_{ex}=330$ nm, $\lambda_{em}=440$ nm).

resides in the liquid phase and decreases the retention of the amino acid and the enantioselectivity. These findings suggest that the affinity of the diastereomeric complexes for the stationary phase is the main factor involved in the chiral recognition process with these types of ligands.

For each amino acid, the optimum separation

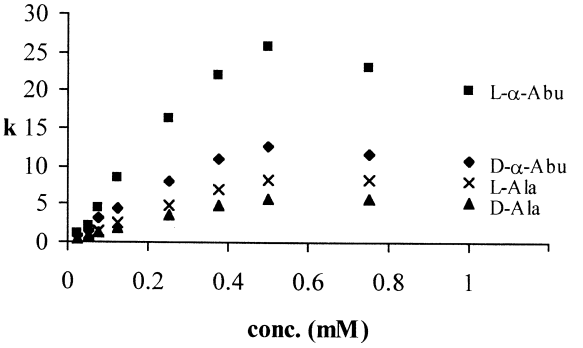


Fig. 5. Effect of selector concentration on the capacity factors (k) of D- and L-alanine and D- and L-α-aminobutyric acids. Conditions: eluent, Me_2PheNN-2–Cu^{II} (1:1) in water–methanol (85:15, v/v), pH 7.0; column, Spherisorb ODS2 (3 μm, 15×0.46 cm); flow-rate, 1 ml/min; column temperature 40°C, fluorescence detection (post-column derivatization with OPA, $\lambda_{ex}=330$ nm, $\lambda_{em}=440$ nm).

conditions were found, taking into account both the enantioselectivity factors and the retention times: the results are reported in Table 1.

Table 1
Enantiomeric separation of D,L-amino acids with the system Me_2Phe-NN-2–Cu^{II}

Amino acid	k_L	k_D	$\alpha=k_L/k_D$	R_s
Glu[a]	8.29	10.50	0.79	1.03
Asp[a]	5.27	7.80	0.68	1.29
Ser[b]	2.75	3.58	0.77	1.22
Thr[b]	2.27	3.42	0.66	1.33
Ala[b]	10.49	3.83	2.74	2.89
Abu[c]	4.11	1.98	2.08	2.22
Val[c]	10.87	6.73	1.62	1.80
Met[c]	16.06	12.69	1.27	1.40
NVal[c]	10.82	7.06	1.53	1.78
Leu[d]	6.62	4.08	1.62	2.67
Tyr[e]	14.19	10.54	1.35	1.35
His[e]	0.77	1.47	0.52	3.11
Phe[f]	6.77	5.95	1.14	1.10
Trp[f]	11.30	9.30	1.22	1.15

Conditions: eluent, 0.5 m*M* Me_2PheNN-2 and 0.5 m*M* copper(II) acetate in water, pH 6.5; column, Spherisorb ODS2 (3 μm, 15×0.46 cm); flow-rate, 0.5 ml/min; room temperature; fluorescence detection (post-column derivatization with OPA, $\lambda_{ex}=330$ nm, $\lambda_{em}=440$ nm); if not otherwise stated. [a]Eluent, 2 m*M* Me_2PheNN-2 and 2 m*M* copper(II) acetate in water–acetonitrile (95:5), pH 7.0. [b]Eluent, 1 m*M* Me_2PheNN-2 and 1 m*M* copper(II) acetate in water, pH 7.5. [c]Column temperature 52°C, [d]eluent, water–methanol (70:30, v/v), pH 7; flow-rate = 1 ml/min; [e]eluent, water–methanol (80:20, v/v); [f]eluent, water–methanol (80:10, v/v), pH 5.5.

With the separation conditions slightly different for each amino acid, it is not proper to compare the separation factors as far as the amino acid side chains are concerned. Indeed, very good results were obtained for most amino acids.

The general scheme proposed [18] for ligand exchange chromatography with chiral additives to the eluent involves partition equilibria of the chiral selector (CuL), the analyte (A) and the diastereomeric complexes between the mobile and the stationary phase (Fig. 6).

According to this model, the overall enantioselectivity of the system turns out to be a complex function, involving the affinity of the different species for either phases and the relative stabilities of the diastereomeric complexes in solution and in the stationary phase. In order to understand the mechanism of chiral separation with our ligands, (i) first, we investigated the copper(II) complexes present in solution in the pH range used in HPLC (5.5–7.5); (ii) then, we determined the stability constants of the ternary complexes with several amino acids in solution (K^{m}_{CuLA}); (iii) finally, we studied the adsorption of the selector on the stationary phase and the enantiomeric separation of amino acids under these conditions.

3.2. Binary Cu^{II} complexation equilibria of PheNN-2 and Me_2PheNN-2 in aqueous solution

The copper(II) complexes formed by the two ligands (L) were previously investigated by potentiometry and were found to be $[CuLH]^{3+}$, $[CuL]^{2+}$, $[Cu_2L_2H_{-2}]^{2+}$ and $[CuLH_{-2}]$ for PheNN-2 [43] and $[CuLH]^{3+}$, $[Cu_2L_2H_{-2}]^{2+}$ and $[CuLH_{-2}]$ for Me_2PheNN-2 [39]. The complexes of the latter ligand are less stable than those of the former, owing to the steric hindrance exerted by the methyl groups.

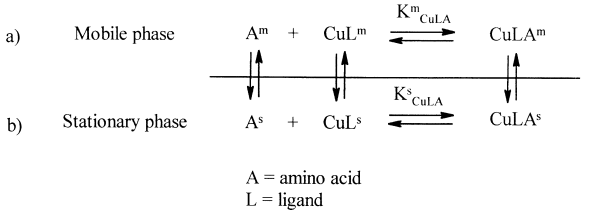


Fig. 6. Schematic representation of formation and partition equilibria in ligand exchange systems with chiral mobile phases.

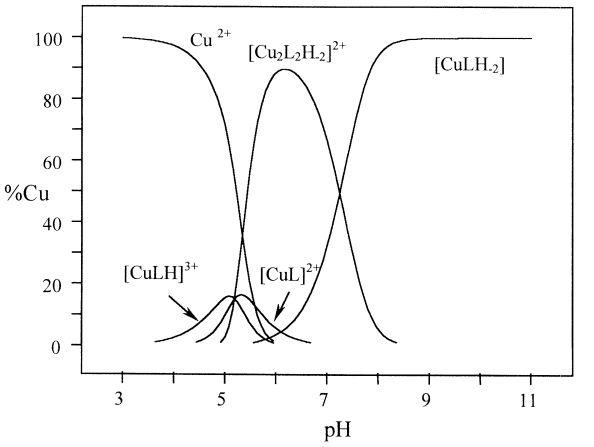


Fig. 7. Species distribution for the system PheNN-2–Cu^{II} (1:1), $c_{Cu}=1$ m*M*.

This effect is particularly remarkable for the dinuclear species $[Cu_2L_2H_{-2}]^{2+}$, which shows a much lower stability for Me_2PheNN-2 (log $\beta=1.81$ for **1b** against log $\beta=5.78$ for **1a**). Thus, with PheNN-2 the predominant species are $[Cu_2L_2H_{-2}]^{2+}$ in the pH range 5.5–7.5 (80% of total copper) and $[CuLH_{-2}]$ at pH>7.5 (100%) (Fig. 7). In contrast, for Me_2PheNN-2 the predominant species is $[CuLH_{-2}]$ (100%) at pH>6.5 (Fig. 8).

The proposed molecular structures of the complexes potentially involved in enantiomeric separation are shown in Fig. 9. The structure of the dinuclear cation

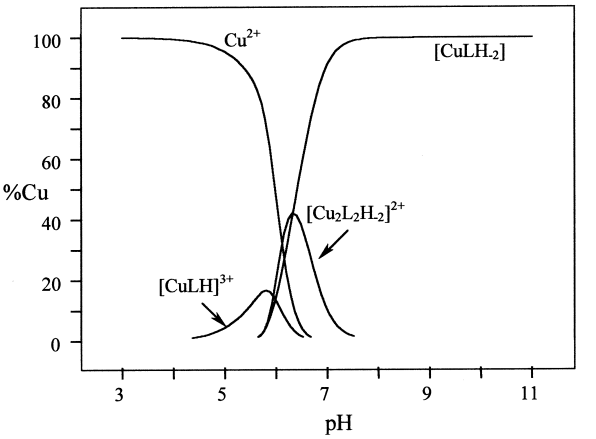


Fig. 8. Species distribution for the system Me_2PheNN-2–Cu^{II} (1:1), $c_{Cu}=1$ m*M*.

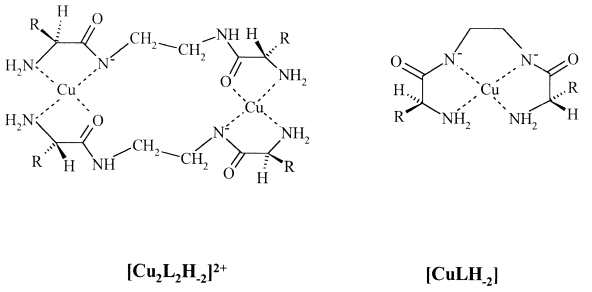


Fig. 9. Proposed structures of binary complexes $[Cu_2L_2H_{-2}]^{2+}$, $[CuLH_{-2}]$ for PheNN-2 and Me_2PheNN-2.

of PheNN-2 has been confirmed by X-ray analysis of the complex $[Cu_2L_2H_{-2}]Cl_2 \cdot 12H_2O$ [39].

3.3. Ternary Cu^{II} complexation equilibria with PheNN-2 and with Me_2PheNN-2 and D- or L-amino acids in aqueous solution

The formation of Cu^{II} ternary complexes with either ligands and some D- or L-amino acids (Val, Phe, Pro, and Trp with PheNN-2 and Phe, Trp, Glu with Me_2PheNN-2) were studied by potentiometry at $T=25$°C and $I=0.1$ *M* (KCl). For calculating the stability constants of the ternary complexes, protonation and Cu^{II} complexation constants of the amino acids were taken from the literature [44].

PheNN-2 forms three mixed species with all the amino acids (A) examined, i.e., $[CuLA]^+$, $[CuLH_{-1}A]$, $[CuLH_{-2}A]^-$ in the pH range 5–11. In contrast, Me_2PheNN-2 does not form the $[CuLH_{-2}A]^-$ complex with D- and L-Glu, while with L-Trp precipitation occurs at pH ca. 7.5. The stability constants obtained are reported in Tables 2 and 3 for PheNN-2 and Me_2PheNN-2, respectively.

The mixed complexes of Me_2PheNN-2 are remarkably less stable than those of PheNN-2 due to the hindrance of the methyl groups on the phenylalanyl moiety and parallel the behavior of the corresponding binary complexes [39]. As the stereoselectivity is concerned, the data reported for PheNN-2 show an appreciable stability difference only for the $[CuLH_{-2}A]^-$ complexes of D- and L-Pro

Table 2
Formation constants (log β_{pqrs}; $\beta_{pqrs}=[Cu_pL_qA_rH_s]/[Cu]^p[L]^q[A]^r[H]^s$) of the ternary Cu^{II} complexes of (*S,S*)-PheNN-2 (L) with D- or L-amino acids (A)

Species	Val		Phe		Pro		Trp	
	D	L	D	L	D	L	D	L
$[CuLA]^+$	13.56 (4)	13.54 (6)	13.58 (3)	13.54 (3)	14.37 (2)	14.36 (2)	14.30 (2)	14.33 (2)
$[CuLH_{-1}A]$	6.75 (2)	6.84 (3)	6.67 (3)	6.64 (2)	7.53 (1)	7.53 (1)	7.17 (2)	7.20 (3)
$[CuLH_{-2}A]^-$	−2.65 (3)	−2.56 (4)	−2.45 (3)	−2.54 (3)	−3.16 (2)	−3.05 (1)	−2.40 (3)	−2.35 (3)
s^{2a}	2.62	2.40	2.59	2.19	0.78	1.67	1.82	3.50
n^a	203	138	208	220	153	293	202	192

$T=25$°C and $I=0.1M$ (KCl). Standard deviations are given in parentheses.
[a] $s^2=\Sigma w_i(E_i^{obs}-E_i^{calc})^2/(n-m)$ = sample variance; $w_i=1/\sigma_i^2$, where σ_i is the expected error on each experimental observation (E_i^{obs}); n = number of observations; m = number of parameters refined.

Table 3
Formation constants (log β_{pqrs}; $\beta_{pqrs}=[Cu_pL_qA_rH_s]/[Cu]^p[L]^q[A]^r[H]^s$) of the ternary Cu^{II} complexes of (*S,S*)-Me_2PheNN-2 (L) with D- or L-amino acids (A)

Species	Glu		Phe		Trp	
	D	L	D	L	D	L
$[CuLA]^+$	12.54 (2)	12.50 (3)	11.89 (9)	11.73(13)	13.06 (1)	13.08 (1)
$[CuLH_{-1}A]$	5.24 (4)	5.06 (5)	5.35 (3)	5.33 (3)	6.02 (1)	6.06 (2)
$[CuLH_{-2}A]^-$	–	–	−4.51 (5)	−4.33 (4)	−4.26 (3)	–
s^2	2.37	2.40	2.56	2.37	1.46	2.13
n	204	213	282	266	391	265

$T=25$°C and $I=0.1$ *M* (KCl). Standard deviations are given in parentheses (s^2 and n as in Table 2).

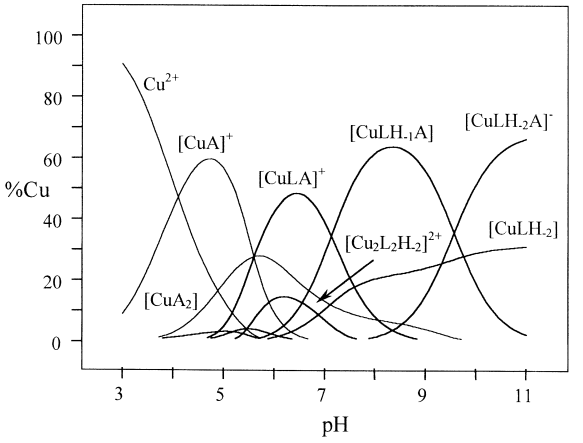


Fig. 10. Species distribution for the system PheNN-2–Cu^{II}–D-Trp (1:1.5:1.5), $c_{Cu}=1$ m*M*.

[$\Delta\log\beta=\log\beta_D-\log\beta_L=-0.11$ (2)], whereas for Me_2PheNN-2 the $\Delta\log\beta$ values presented by Glu ($[CuLH_{-1}A]$, 0.18 (6)) and by Phe ($[CuLH_{-2}A]^-$, −0.18 (6)) are not significant.

A representative distribution diagram for the mixed complexes of PheNN-2 and D-Trp is shown in Fig. 10: $[CuLA]^+$ is formed between pH 5 and 8 with a maximum of ca. 50% of total copper at pH 6.5; $[CuLH_{-1}A]$ starts to be formed at pH 6 and reaches a maximum of ca. 60% at pH 8.3; $[CuLH_{-2}A]^-$ begins to be formed at pH ca. 8.5 reaching 70% at pH 11 (and it is outside the pH range of the eluents used in the chromatographic system).

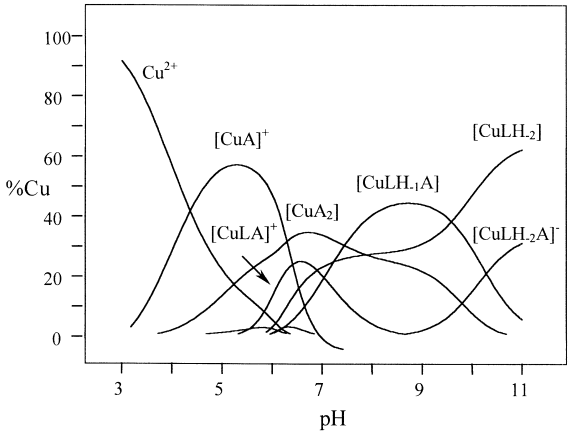


Fig. 11. Species distribution for the system Me_2PheNN-2–Cu^{II}–D-Trp (1:1.5:1.5), $c_{Cu}=1$ m*M*.

The distribution diagram for Me_2PheNN-2 and D-Trp (Fig. 11) shows that the mixed species are present in notably lower amount with respect to PheNN-2: $[CuLA]^+$ (25%, pH 6.8), $[CuLH_{-1}A]$ (45%, pH 8.5), $[CuLH_{-2}A]^-$ (32%, pH 11).

In order to confirm the formation of these complexes, we studied the ternary system Cu^{II}–Me_2PheNN-2–D-Trp also by ESI-MS, performing the experiments in the positive ion mode at pH 7.0. The species $[CuLA]^+$ (m/z 648) and $[CuLH_{-1}A]$ (as $[CuLH_{-1}A]K^+$ at m/z 686) were detected, together with the binary complexes $[CuLH_{-2}]$ (as $[CuLH_{-2}]K^+$, m/z 482) and $[CuA_2]$ (as $[CuA_2]K^+$, m/z 508). The presence of the negatively charged species $[CuLH_{-2}A]^-$ (m/z 646) was confirmed at pH 11.6 in the negative ion mode.

The molecular structures proposed for the ternary complexes of both PheNN-2 and Me_2PheNN-2 are reported in Fig. 12. $[CuLA]^+$ may be considered as square planar with L chelating Cu^{II} by an amino nitrogen and a carbonyl oxygen, while the amino acid occupies the two remaining equatorial positions. We cannot, however, exclude an eventual interaction of the other amino group of the ligand at the axial position, as well as axial coordination of water molecules.

The tetracoordination of the mixed complex $[CuLA]^+$ of PheNN-2 is supported by the stability constant value of the stepwise equilibrium:

$$[CuL]^{2+} + A^- = [CuLA]^+$$

($\log K=7.22$ for A=L-Phe) which is similar to that obtained for the corresponding bidentate ligand (*S*)-phenylalaninamide (Phe-NH_2) ($\log K=7.29$ for A=L-Phe) [31].

Very likely, the species $[CuLH_{-1}A]$ has the same geometry, with the ligand chelating via an amino and a deprotonated amido nitrogen. Actually, the equilibrium constant of the reaction:

$$[CuLA]^+ = [CuLH_{-1}A] + H^+$$

($\log K=-6.90$) is in agreement with that of the bidentate $PheNH_2$ ($\log K=-6.70$), both ligands changing from a (*N*,*O*) to a (*N*,N^-) coordination.

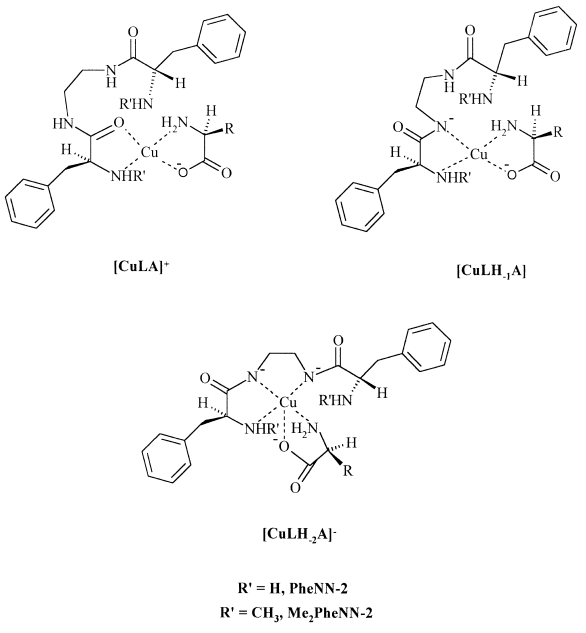


Fig. 12. Proposed structures of the ternary complexes of Cu^{II} with PheNN-2 and Me_2PheNN-2 and L-amino acids.

Also in this case, we cannot exclude an eventual axial interaction of the other amino group of the ligand.

As regards $[CuLH_{-2}A]^-$, the ligand is most probably terdentate (N,N^-,N^-) in the equatorial plane, the fourth position being occupied by the amino group of the amino acid and one axial position by the carboxylato oxygen. In fact, the log K values of the equilibrium:

$$[CuLH_{-2}] + A^- = [CuLH_{-2}A]^-$$

for PheNN-2 (log $K = 3.33$, A = L-Phe) and Me_2PheNN-2 (log $K = 2.60$, A = L-Phe) are similar to that reported for the terdentate ligand PheN-2 (log $K = 3.71$, A = L-Phe) for the reaction:

$$[CuLH_{-1}]^+ + A^- = [CuLH_{-1}A]$$

The lower values obtained for the tetradentate ligands may be attributed to the fact that the displacement of an amino group from the equatorial position is required for the coordination of the amino acid. The even lower stability of $[CuLH_{-2}A]^-$ for Me_2PheNN-2 might be due to steric hindrance of the methyl groups on the phenylalanine moiety.

Actually, the possibility that a tetradentate ligand, such as the bideprotonated PheNN-2, might act also as terdentate is not unusual; in fact for $[Cu(trien)]^{2+}$ a mixture of terdentate and tetradentate species has been suggested to be present in solution on the basis of spectroscopic data [45].

3.4. Enantiomeric separation of amino acids by HPLC with the selector adsorbed on the stationary phase

In order to gain evidence about the role played by the stationary phase, we performed enantiomeric separation with the chiral selector adsorbed on the stationary phase by eluting with bidistilled water: in these conditions, good enantiomeric separations of amino acids were obtained (Table 4).

The retention times increased by continuously eluting with water for more than a week (D,L-Ala, first injection, $t_D = 9.61$, $t_L = 17.14$; after 1 day, $t_D = 12.77$ min, $t_L = 22.23$ min; after 1 week, $t_D = 13.25$ min, $t_L = 22.97$ min), while the enantioselectivity factors did not change ($\alpha = 1.7–1.8$).

Probably, when elution with water is commenced, there is an excess of copper complex in the chromatographic system, which forms a CuA-type complex in solution which does not interact with the adsorbed ligand. Later, the separation is performed mainly by the selector adsorbed on the column. By continuously eluting with water for a month, the

Table 4
Enantiomeric separation of D,L-amino acids with the system Me_2Phe-NN-2–Cu^{II} adsorbed on the column stationary phase

Amino acid	k_L	k_D	$\alpha = k_L/k_D$	R_s
Ser[a]	1.58	2.12	0.75	1.13
Thr[a]	3.32	4.01	0.83	1.21
Ala[a]	7.14	3.90	1.83	1.57
Abu[b]	1.28	0.61	2.10	2.17
Val[b]	2.68	1.71	1.57	2.25
Tyr[b]	3.52	2.88	1.22	1.30

Conditions: solution for adsorption, 0.5 mM Me_2PheNN-2 and 0.5 mM copper(II) acetate in water, pH 7.5; [a]eluent, water, pH 7.5; flow-rate = 1 ml/min; column temperature, 35°C; column, Spherisorb ODS2 (3 μm, 15×0.46 cm); fluorescence detection (post-column derivatization with OPA, $\lambda_{ex} = 330$ nm, $\lambda_{em} = 440$ nm); [b]pH 6, other conditions as in [a].

selector and the copper ion started to be removed from the column, so that the capacities and the enantioselectivity decreased.

3.5. Mechanism of chiral separation

It is now possible to discuss the mechanism of chiral separation with copper(II) complexes of tetradentate ligands added to the eluent in the light of the present results and on the basis of the scheme reported in Fig. 6. First, we can consider the equilibria in the mobile phase (Fig. 6a).

The copper(II) complexes of PheNN-2 formed in the pH range examined, which potentially may perform chiral separation are $[CuL]^{2+}$, $[Cu_2L_2H_{-2}]^{2+}$ and $[CuLH_{-2}]$. However, $[CuL]^{2+}$ appears to be a poor selector since it is present at a very low concentration (maximum ~5% at pH 5.5). Most probably, in this case, the main selector is $[Cu_2L_2H_{-2}]^{2+}$, which is the major species at pH 6.0, used for the chromatographic separation, and which may undergo a ligand exchange equilibrium, forming $[CuLH_{-1}A]$ or $[CuLA]^{+}$.

Instead, in the case of Me_2PheNN-2, the dinuclear species is a minor one and the major species present in solution is $[CuLH_{-2}]$. This complex, which may undergo ligand exchange more easily than in the case of PheNN-2 on account of its lower stability, may give rise to the ternary complex $[CuLH_{-1}A]$ by partial decomplexation of one arm of the ligand with reprotonation of the amido nitrogen and coordination of the amino acid at the two free equatorial positions (Fig. 12).

To summarize, $[CuLH_{-1}A]$ seems to be the main candidate to account for chiral separation, as the highest enantioselectivity was observed in the pH range in which $[CuLH_{-1}A]$ is the main ternary species (pH 7–7.5). It may be formed according to the following equilibria:

$$[CuLA]^{+} = [CuLH_{-1}A] + H^{+}$$

$$[Cu_2L_2H_{-2}]^{2+} + 2A^{-} = 2[CuLH_{-1}A]$$

$$[CuLH_{-2}] + HA = [CuLH_{-1}A]$$

However, we do not have evidence concerning the kinetics of the ligand exchange.

In a pH range not involved in the chromatographic resolution (pH>8.5), the species $[CuLH_{-2}]$ appears to be able to interact with the amino acid giving rise to $[CuLH_{-2}A]^{-}$. This species might derive also from $[CuLH_{-1}A]$ upon proton loss from the second amide nitrogen and coordination with the amino acid:

$$[CuLH_{-2}] + A^{-} = [CuLH_{-2}A]^{-}$$

$$[CuLH_{-1}A] = [CuLH_{-2}A]^{-} + H^{+}$$

However, all these equilibria in solution turned out to be non enantioselective. These results allow to exclude that equilibria (a) in Fig. 6 are relevant for chiral discrimination and confirm the previously reported hypothesis that chiral separation does not occur in the mobile phase, but rather at the solid–liquid interphase or following partition equilibria of the ternary complexes between the liquid and the solid phase [29,34].

The experiments performed with the selector Me_2PheNN-2–Cu^{II} adsorbed on the stationary phase support this conclusion as a very good enantioselectivity was observed, without addition of the selector to the liquid phase, up to more than 1 month. Therefore, it appears that indeed in this case the diastereomeric complexes are formed directly on the stationary phase.

4. Conclusions

With the two tetradentate selectors, ligand exchange does occur by displacement of two binding sites. In the case of PheNN-2, the selector most probably is the species $[Cu_2L_2H_{-2}]^{2+}$, which may undergo ligand exchange at either complexation sites, forming complexes of the $[CuLH_{-1}A]$ or $[CuLA]^{+}$ type. With Me_2PheNN-2, at pH>6.5 $[CuLH_{-2}]$ appears to be the unique species present and most probably $[CuLH_{-1}A]$ is the ternary species involved in chiral separation also on account of the fact that it is a neutral species with higher affinity for the apolar stationary phase. This hypothesis is supported by the elution order of the amino acids, which is the same as that observed for the bidentate ligand MePhe–NH_2 [29], and by the capacity factors which increased very much at pH 7–7.5 (Fig. 4).

The very good enantioselectivity observed with these tetradentate ligands may be explained by the fact that they undergo ligand exchange forming stable mixed complexes, in which the amino acidato is coordinated at two equatorial positions. Moreover, these ligands are lipophilic, so that they are easily adsorbed on the column and may give rise to crowded ternary complexes. Finally, they have a second chiral center in the proximity of the binding site, which can provide further discriminating interactions.

In conclusion, with these chiral copper(II) complexes added to the mobile phase, we believe that chiral discrimination is partially due to the formation of the diastereomeric complexes directly on the phase and partially to the different affinities for the stationary phase of the neutral diastereomeric ternary species $[CuLH_{-1}A]$ formed in solution.

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