CHROM. 25 503

Chiral separation of unmodified amino acids by ligand-exchange high-performance liquid chromatography using copper(II) complexes of L-amino acid amides as additives to the eluent

Gianni Galaverna, Roberto Corradini, Eriberto de Munari, Arnaldo Dossena and Rosangela Marchelli*

Dipartimento di Chimica Organica e Industriale dell' Università, Viale delle Scienze, I-43100 Parma (Italy)

ABSTRACT

Copper(II) complexes of L-amino acid amides added to the eluent in reversed-phase high-performance liquid chromatography are able to perform chiral discrimination of unmodified amino acids with high enantioselectivity. The mechanism is consistent with a ligand exchange between the binary initial copper(II) complex and the enantiomers. Evidence is provided that the exchange of the ligand is actually occurring during the chromatographic separation. The system involves a series of equilibria of exchange in the aqueous solution, in the stationary phase and between the two phases. Enantioselectivity is essentially due to the adsorption of the diastereomeric ternary species on the column, whereas the relative stabilities of the mixed complexes in the mobile phase seem to be negligible with respect to the overall discrimination process. The structural features of the initial copper complexes greatly affect the stereoselectivity of the process. The chromatographic parameters (pH, selector concentration, eluent polarity, ionic strength) are examined.

INTRODUCTION

Chiral separation of amino acids is of great interest in many research fields, from geochronological to pharmaceutical and biomedical areas [1–3]. Recently, we have found surprisingly large amounts of D-amino acids in foods, not only in connection with heat or alkali treatments, but also with the most common fermentation processes, such as cheese and yoghurt preparations [4]. Whether D-amino acids are derived from the lysis of the bacterial cell walls and/or other microbiological transformations is an intriguing question, as also is their biological significance at the nutritional, toxicological and organoleptic levels [5].

In the last decade, the enantiomeric separation of unmodified amino acids by HPLC has received much attention. In particular, ligand-exchange chromatography (LEC) [6] has proved to be successful with a variety of copper(II) complexes of amino acids or amino acid derivatives either chemically bonded to the stationary phases [7–9] or as additives to the eluent [10–23]. The addition of chiral metal complexes to the eluent is particularly useful in analytical applications, being very easy to handle, flexible, and efficient [24]. In fact, achiral columns can be used in combination with different optically active complexes with high enantioselectivity and efficiency.

Several years ago [25–27] we started an exten-

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* Corresponding author.

sive investigation on the mechanism of chiral discrimination by ligand exchange with copper(II) complexes of bidentate, tridentate and tetradentate ligands of the amino amide type. In fact, whether chiral discrimination occurs via a total or partial displacement of one ligand from a binary complex is still a matter of discussion. Alternative mechanisms have been proposed, involving also apical and outer-sphere interactions [28]. In particular, in a previous paper [26] we reported that with copper(II) complexes of bidentate ligands (L-amino acid amides) added to the mobile phase, very good chiral separations of D,L-dansyl (Dns)-amino acids in reversed-phase HPLC were obtained. We now report that copper(II) complexes of the same (or slightly modified) ligands when added to the eluent are able to perform chiral separations of unmodified amino acids in reversed-phase HPLC with either postcolumn derivatization with o-phthalaldehyde (OPA) and fluorescence detection or with UV detection. The effects of ligand hydrophobicity and structure, complex concentration, pH, solvent polarity and ionic strength were investigated.

L-Amino acid amides are commercially available or can be easily synthesized [29], with retention of configuration. Their ability to form copper(II) complexes, both in aqueous solution and in the solid state, was also studied. The distribution pattern of the various complexes existing at different pHs and their thermodynamic stabilities were established by potentiometry and spectroscopy [30]. The crystal structures of several copper complexes with L-phenylaninamide, L-N^2-methyl- and L-N^2,N^2-dimethylphenylalaninamide [31] and L-prolinamide [32] were determined by X-ray crystallography and may provide important clues regarding the eventual interactions involved in chiral discrimination. Whether the mechanism of chiral discrimination proceeds via ligand exchange in the chromatographic system was investigated by using copper(II) complexes of L-tryptophanamide.

EXPERIMENTAL

Equipment

Chromatographic analyses were performed on a Waters Model 440 chromatograph equipped with a Model 420 fluorescence detector and a Waters 745 data module or with a UV detector set at 254 or 280 nm. A C$_{18}$ Spherisorb ODS-2 (3 μm) column (150 × 4.6 mm I.D.) was used.

Synthesis of ligands

N^2-Methyl-L-amino acid amide hydrochlorides. N-Methylamino acids were synthesized from Cbz-amino acids using McDermott and Benoiton's procedure [33] or were obtained from Sigma (St. Louis, MO, USA). The amidation procedure was performed according to an original method developed in our laboratory [29] by reacting the N-hydroxysuccinimidyl ester with diaminomethane dihydrochloride in dioxane. Compounds synthesized were N^2-methyl-L-phenylalaninamide hydrochloride (L-MePhe-A · HCl) [30] and N^2-methyl-L-valinamide hydrochloride (L-MeVal-A · HCl) [29].

N^2,N^2-Dimethyl-L-amino acid amide hydrochlorides. These ligands were obtained from the corresponding L-amino acid amide hydrochlorides and formaldehyde in methanol by stirring under hydrogen in the presence of 10% Pd–C at 40°C for 3 h. Compounds synthesized were N^2,N^2-dimethyl-L-phenylalaninamide hydrochloride (L-Me$_2$Phe-A · HCl) [30] and N^2,N^2-dimethyl-L-valinamide hydrochloride (L-Me$_2$Val-A · HCl). Data for the latter compound were as follows: yield, 85%; m.p., 262–264°C; $[\alpha]_D^{25°C} = +24.3$ ($c = 1$, 95% EtOH). IR (KBr): $\nu = 3310$, 3150, 2980, 1690 cm^{-1}. ^{1}H NMR (C^{2}HCl$_3$, free amine): δ 1.0 (dd, 6H, 2 CH$_3$), 2.0–2.2 (m, 1H, CH$_\beta$), 2.3 [s, 6H, N(CH$_3$)$_2$], 2.45 (d, 1H, CH$_\alpha$), 5.7 (broad s, 1H, CONH), 6.3 (broad s, 1H, CONH) ppm. ^{13}C NMR (C^{2}HCl$_3$, free amine): δ 18 (CH$_3$), 20 (CH$_3$), 28 (CH$_\beta$), 43 [N(CH$_3$)$_2$], 75 (CH$_\alpha$), 195 (CO) ppm. Mass spectrum: m/z

144 (traces, M^+), 100 (100), 85 (38), 70 (18), 56 (10).

Preparation of derivatizing agent

Boric acid (18.55 g) was dissolved in doubly distilled water (1 l) and the pH was adjusted to 10 by addition of solid KOH (pellets). EDTA (2.5 g) was added and the pH was readjusted to 10. OPA (0.8 g) was dissolved in mercapto-ethanol (4 ml) and then added to the former solution with a syringe under vigorous magnetic stirring. The resulting solution was filtered and degassed under reduced pressure on HPLC filters (0.45 μm; Millipore).

Mobile phase preparation

The ligand and copper acetate were carefully weighed to give the ratios required and dissolved in water or in a water–organic solvent (methanol or acetonitrile) mixture. When needed, sodium acetate (0.1–0.3 M) was added. The solutions were adjusted to the desired pH with acetic acid or sodium hydroxide, then filtered and degassed under reduced pressure. Before starting the analysis, the mobile phase was allowed to flow until reproducible retention times were obtained. The column efficiency was checked by calculating the number of theoretical plates per metre; 25 000–50 000 plates per metre were obtained, depending on the amino acid and on the selector. The column dead volume (V_0) was determined from the elution time of an unretained solute (acetic acid).

Ligand-exchange experiment

L-Tryptophanamide (L-Trp-A) and copper(II) acetate were dissolved in water in the appropriate ratio (2:1) to obtain a 0.2 mM complex concentration. The pH was adjusted at 5.8 and the solution was filtered and degassed under reduced pressure on HPLC filters (0.45 μm). The mobile phase was allowed to flow on a C_{18} Spherisorb 3 ODS-2 column and, after equilibration (2 h), the analysis was started. A fluorescence detector, set at the excitation and emission wavelengths of tryptophan ($\lambda_{exc.} = 295$ nm, $\lambda_{em.} = 359$ nm), or a UV detector, set at 254 or 280 nm, was used.

RESULTS AND DISCUSSION

We utilized copper(II) complexes of L-prolinamide (L-Pro-A), L-phenylalaninamide (L-Phe-A), N^2-methyl-L-phenylalaninamide (L-MePhe-A), N^2,N^2-dimethyl-L-phenylalaninamide (L-Me$_2$Phe-A), L-valinamide (L-Val-A), N^2-methyl-L-valinamide (L-MeVal-A), N^2,N^2-dimethyl-L-valinamide (L-Me$_2$Val-A) and L-tryptophanamide (L-Trp-A) as additives to the eluent. All ligands were easily prepared in high yield and optical purity [29].

Chromatographic results

Copper(II) complexes of L-Phe-A and L-MePhe-A in aqueous solution in the pH range 5–7.5 in reversed-phase HPLC proved to be enantioselective towards polar and non-polar amino acids, except glutamic acid and serine. All other amides were able to separate only non-polar amino acids. Results are reported in Tables I and II and in Figs. 1 and 2.

Effect of ligand hydrophobicity

By taking the hydrophobic parameters π for the amino acid side-chains [34] as a measure of the lipophilicity of the selectors and selectands, it appears that the more lipophilic selectors Phe-A and MePhe-A ($\pi_{Phe} = 1.63$) gave the best enantioselectivity factors with most amino acids, whereas the least lipophilic Pro-A ($\pi_{Pro} = 0.77$) gave very good results only with non-polar amino acids. The analogous MeVal-A ($\pi_{Val} = 1.27$) gave very poor results.

Effect of N-substitution on the amino group of the ligand

The introduction of one methyl group on the amino nitrogen of the ligand improved the enantioselectivity, whereas the presence of a second methyl group enhanced the separation factors of non-polar amino acids, but it prevented the separation of the polar compounds. Accordingly, the best separation factors with non-polar amino acids were obtained with the more hindered ligand Me$_2$Phe-A and with Pro-A, which has a rigid cyclic moiety. Polar amino acids were separated only by Phe-A and MePhe-A, which

TABLE I

CHIRAL SEPARATION OF AMINO ACIDS WITH COPPER(II) COMPLEXES OF L-AMINO ACID AMIDES: CAPACITY FACTORS (k'), ENANTIOSELECTIVITY FACTORS ($\alpha = k'_L/k'_D$) AND HYDROPHOBIC CONSTANTS (π) FOR AMINO ACID SIDE-CHAINS

Conditions: 2 mM amino acid amide–1 mM copper (II) acetate; pH 6.0; column, Spherisorb 3 ODS-2 (3 μm, 15 × 0.46 cm I.D.); fluorescence detection (postcolumn derivatization with OPA); UV detection at 254 nm for Pro; flow-rate, 0.5 ml/min; room temperature; t_0 = 3.24 min (retention time of CH_3COOH).

Amino acid	π	Phe-A			MePhe-A			Me$_2$Phe-A		
		k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α
Glu	−0.98	6.54	6.54	1.00	2.40	2.40	1.00	0.58	0.58	1.00
Asp	−1.05	1.65	1.88	0.88	1.28	1.57	0.82	0.35	0.35	1.00
Ser	−0.08	0.85	0.85	1.00	0.20	0.20	1.00	0.06	0.06	1.00
Thr	0.33	1.23	1.46	0.84	0.29	0.52	0.56	0.13	0.13	1.00
His	−0.40	2.62	2.62	1.00	0.84	1.68	0.50	0.14	0.38	0.37
Ala	0.40	1.62	1.38	1.17	0.54	0.37	1.50	0.23	0.12	1.92
α-NBu	0.83[a]	4.38	3.38	1.30	2.33	1.46	1.60	1.24	0.49	2.53
Met	1.42	20.92	16.54	1.27	12.84	9.77	1.31	6.81	4.28	1.59
Val	1.18	16.77	9.08	1.85	7.61	4.31	1.77	4.24	1.77	2.40
Leu	1.64	47.69	31.31	1.52	33.74	18.41	1.83	12.77	6.05	2.11
Pro	0.77	17.54	14.46	1.21	3.75	0.82	4.57	2.41	0.50	4.82
Nval	1.37	17.85	11.15	1.60	5.96	3.19	1.87	4.33	1.96	2.21
Tyr	0.88	43.00	22.69	1.90	29.04	13.82	2.10	14.95	6.16	2.43

[a] Sum of fragmental contributions π_f [34].

give a good compromise between lipophilicity and steric hindrance. With the latter, a reversal of elution order was observed: L > D for the non-polar and L < D for the polar amino acids.
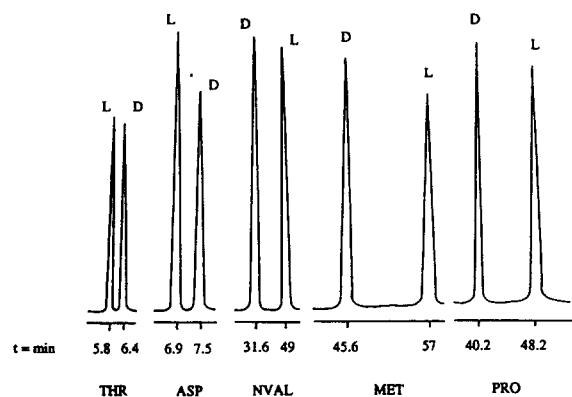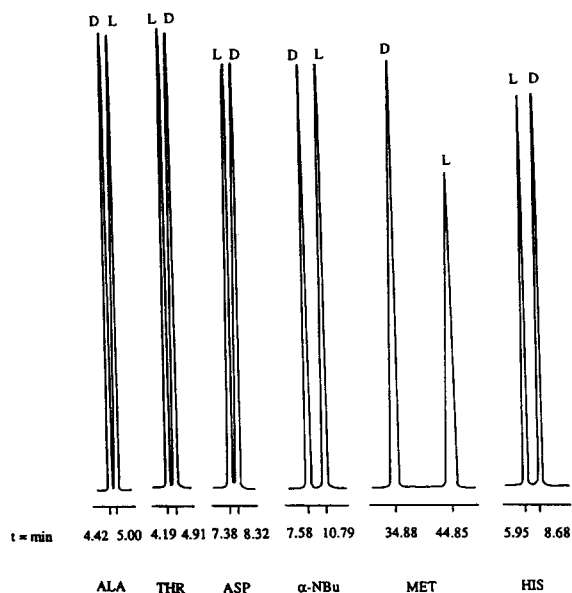

Fig. 1. Chromatographic separation of D,L-amino acids with L-Phe-A–Cu(II). Conditions: 2 mM L-Phe-A, 1 mM copper(II) acetate dissolved in water; pH = 6; column, Spherisorb ODS-2 (3 μm, 15 × 0.46 cm I.D.); room temperature; flow-rate, 0.5 ml/min; UV detection at 254 nm.

Fig. 2. Chromatographic separation of D,L-amino acids with L-MePhe-A–Cu(II). Conditions: 2 mM L-MePhe-A, 1 mM copper(II) acetate dissolved in water; pH 6; column, Spherisorb ODS-2 (3 μm, 15 × 0.46 cm I.D.); room temperature; flow-rate, 0.5 ml/min; fluorescence detection (postcolumn derivatization with OPA).

TABLE II

CHIRAL SEPARATION OF AMINO ACIDS WITH COPPER(II) COMPLEXES OF L-AMINO ACID AMIDES: CAPACITY FACTORS (k'), ENANTIOSELECTIVITY FACTORS ($\alpha = k'_L/k'_D$) AND HYDROPHOBIC CONSTANTS (π) FOR AMINO ACID SIDE-CHAINS

Conditions: 2 mM amino acid amide–1 mM copper (II) acetate; pH 6.0; column, Spherisorb 3 ODS-2 (3 μm, 15 × 0.46 cm I.D.); fluorescence detection (postcolumn derivatization with OPA); UV detection at 254 nm for Pro; flow-rate, 0.5 ml/min; room temperature; t_0 = 3.24 min (retention time of CH_3COOH).

Amino acid	π	Pro-A			MeVal-A			Me$_2$Val-A		
		k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α
Glu	−0.98	0.33	0.33	1.00	0.05	0.05	1.00	0.28	0.28	1.00
Asp	−1.05	0.31	0.31	1.00	0.03	0.03	1.00	0.29	0.29	1.00
Ser	−0.08	0.18	0.18	1.00	0.09	0.09	1.00	0.30	0.30	1.00
Thr	0.33	0.30	0.30	1.00	0.17	0.17	1.00	0.48	0.48	1.00
His	−0.40	0.55	0.71	0.78	0.42	0.63	0.67	1.15	1.15	1.00
Ala	0.40	0.36	0.28	1.29	0.18	0.18	1.00	0.55	0.55	1.00
α-NBu	0.83[a]	1.10	0.54	2.04	0.62	0.62	1.00	1.13	1.03	1.10
Met	1.42	5.90	3.43	1.72	3.13	2.96	1.06	4.19	3.82	1.10
Val	1.18	5.50	1.80	3.05	2.49	2.16	1.15	3.22	2.74	1.18
Leu	1.64	6.55	3.28	2.00	6.48	6.08	1.07	8.33	7.06	1.18
Pro	0.77	5.29	2.21	2.39	2.39	1.85	1.29	3.09	2.25	1.37
NVal	1.37	4.61	2.38	1.94	2.28	2.07	1.10	3.08	2.70	1.14
Tyr	0.88	10.31	6.07	1.70	6.83	6.53	1.05	8.13	6.82	1.19

[a] Sum of fragmental contributions π_f [34].

Effect of pH

Chiral discrimination occurs in the pH range 5.0–7.5. By increasing the pH, the capacity factors of both enantiomers increased, especially that of the most retained enantiomer, so that the selectivity factors also increased, as reported in Fig. 3 for L-Pro-A and in Fig. 4 for MePhe-A.

From potentiometric titrations, we have found [30] that in the pH range considered several copper(II) complexes are present: CuL^{2+}, CuL_2^{2+}, $CuLH_{-1}^+$, $CuL_2H_{-1}^+$ and CuL_2H_{-2}. In Fig. 5 the species distribution for the Cu(II)–MePhe-A system and in Fig. 6 the structures of the complexes are reported.

As shown by Dallavalle et al. [30] and in agreement with Sigel and Martin [35], deprotonation of the amide starts at about pH 5 in the presence of the copper(II) ion. Therefore, it is difficult to ascribe enantioselectivity to a single species, as several equilibria co-exist in the pH range considered. The better enantioselectivity factors (α) obtained at higher pH can be accounted for by the presence of a neutral initial Cu(II) complex or by the formation of neutral ternary species, which may display a better affinity for the column.
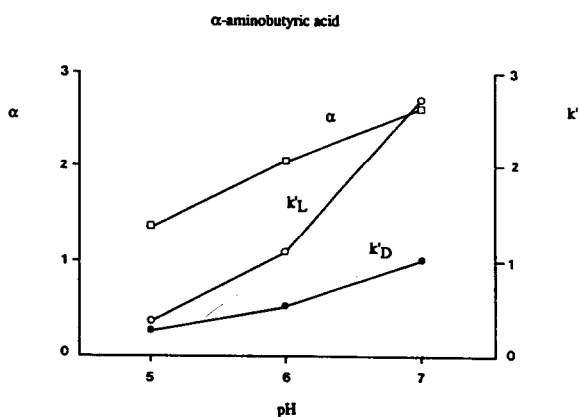

Fig. 3. Variation of the capacity factors (k') and enantioselectivity factors (α) as a function of pH with L-Pro-A–Cu(II). Conditions: 4 mM L-Pro-A, 2 mM copper(II) acetate dissolved in water; room temperature; column, Spherisorb ODS-2 (3 μm, 15 × 0.46 cm I.D.); flow-rate, 0.5 ml/min.
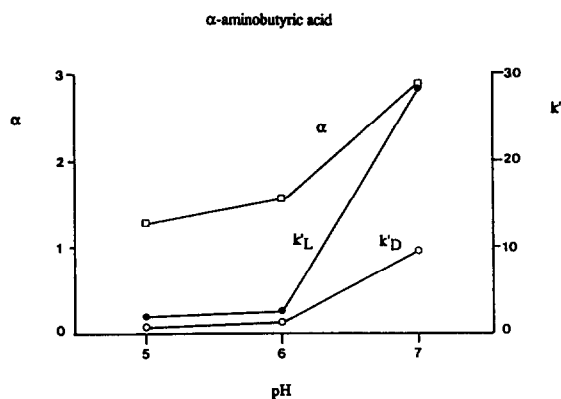
Fig. 4. Variation of the capacity factors (k') and enantio-selectivity factors (α) as a function of pH with L-MePhe-A–Cu(II). Conditions: 4 mM L-MePhe-A, 2 mM copper(II) acetate dissolved in water; room temperature; column, Spherisorb ODS-2 (3 μm, 15 × 0.46 cm I.D.); flow-rate, 0.5 ml/min.

Fig. 6. Structures of the complexes of L-amino acid amides with copper(II).

Effect of amino acid side-chain

We have considered the effect of the length of the amino acid side-chain (R) on the enantio-selectivity factor (α) in the case of Pro-A (Fig. 7): as the length (and therefore the hydrophobic constant) of the linear side chain increased (Ala $< \alpha$-NBu $<$ NVal $<$ NLeu), α also increased, owing to the stronger interaction of the more retained enantiomer with the non-polar C_{18} column (solvophobic effect) (Table II). More-over, branching at C_β (Val, Ile) enhanced the

enantioselectivity factor, on account of an enhanced steric discriminating effect. Branching at further positions (C_γ, Leu) had negligible effects. The same trend was observed with the ligand MePhe-A.
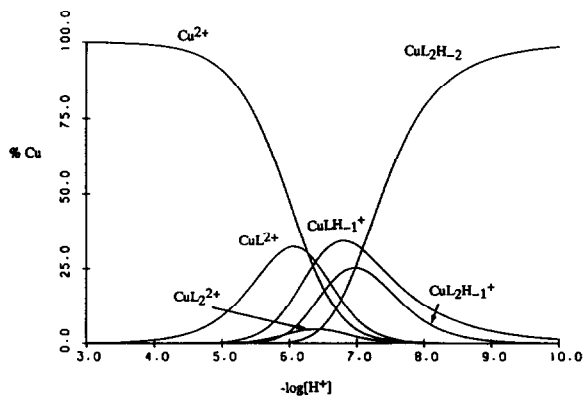

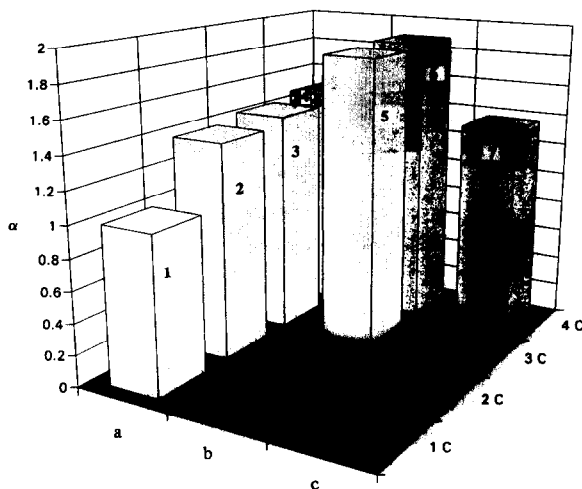Fig. 5. Species distribution for the Cu(II)–MePhe-A system with c_{Cu} = 0.002 M and c_L = 0.004 M as a function of $-\log[\text{H}^+]$.

Fig. 7. Three-dimensional representation of the effect of the side-chain of the amino acids on the enantioselectivity factor (α) with L-Pro-A–Cu(II). 1C, 2C, 3C, 4C = number of carbon atoms in the amino acid side-chain; (a) linear side-chain; (b) β-branching; (c) γ-branching. 1 = Ala; 2 = α-NBu; 3 = NVal; 4 = NLeu; 5 = Val; 6 = Ile; 7 = Leu.

Effect of concentration of initial Cu(II) complexes

The concentration effect was studied with Pro-A and MePhe-A at pH 7 in the concentration range 1–8 mM. As shown in Fig. 5, the main species present at this pH are $CuLH_{-1}^{+}$, $CuL_2H_{-1}^{+}$ and CuL_2H_{-2}. First, the chromatographic system was conditioned with the copper complex at a given molarity for 30 min. Then the experiment was performed with the selector in the mobile phase at the same concentration. The results are different with the selectors L-Pro-A and L-MePhe-A. With the former, the enantioselectivity factor (α) increased from a concentration of 1 mM, reached a maximum at 4 mM and then decreased. In contrast, with the latter the best enantioselectivity factor was obtained at 1 mM. It is evident that a higher concentration of the complex with the less lipophilic Pro-A is needed to saturate the column. The experimental details are reported in Figs. 8 and 9 for the separation of D,L-α-aminobutyric acid.

Effects of eluent polarity and ionic strength

On adding sodium acetate to the eluent both polar and non-polar amino acids were less retained, so that α decreased. The same effect was observed on addition of an organic modifier (acetonitrile) to the aqueous solution containing the chiral copper(II) complex. However, acetonitrile must be used to reduce retention times for the separation of the most lipophilic amino acids
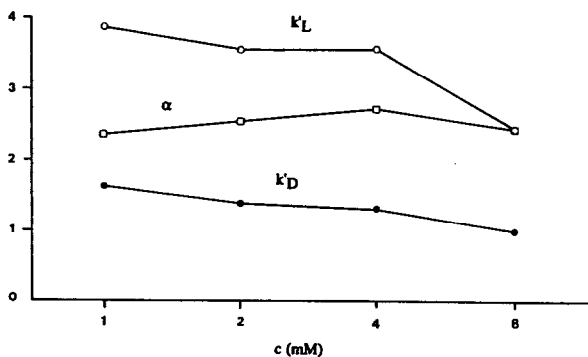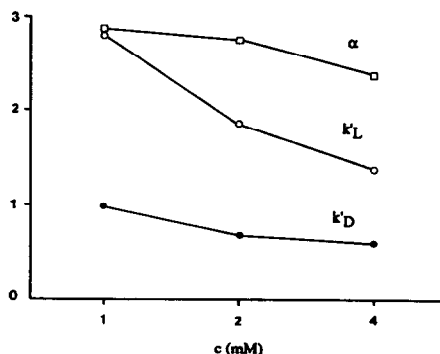

Fig. 9. Variation of the capacity factors (k') and enantioselectivity factors (α) as a function of the initial complex concentration in the eluent: L-MePhe-A–Cu(II); amino acid = α-aminobutyric acid.

such as phenylalanine and tryptophan, as shown in Fig. 10.

Separation of a mixture of amino acids

By using MePhe-A and copper(II) acetate, it was possible to obtain the separation of a mix-
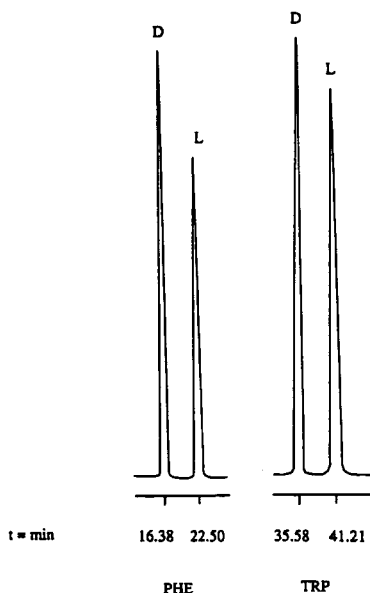

Fig. 8. Variation of the capacity factors (k') and enantioselectivity factors (α) as a function of the initial complex concentration in the eluent: L-Pro-A–Cu(II); amino acid = α-aminobutyric acid.

Fig. 10. Chromatographic separation of D,L-Phe and D,L-Trp with L-MePhe-A–Cu(II). Conditions: 2 mM L-MePhe-A, 1 mM copper(II) acetate; eluent, water–acetonitrile (90:10); pH 6; column, Spherisorb ODS-2 (3 μm, 15 × 0.46 cm I.D.); room temperature; flow-rate, 0.5 ml/min; fluorescence detection (postcolumn derivatization with OPA).
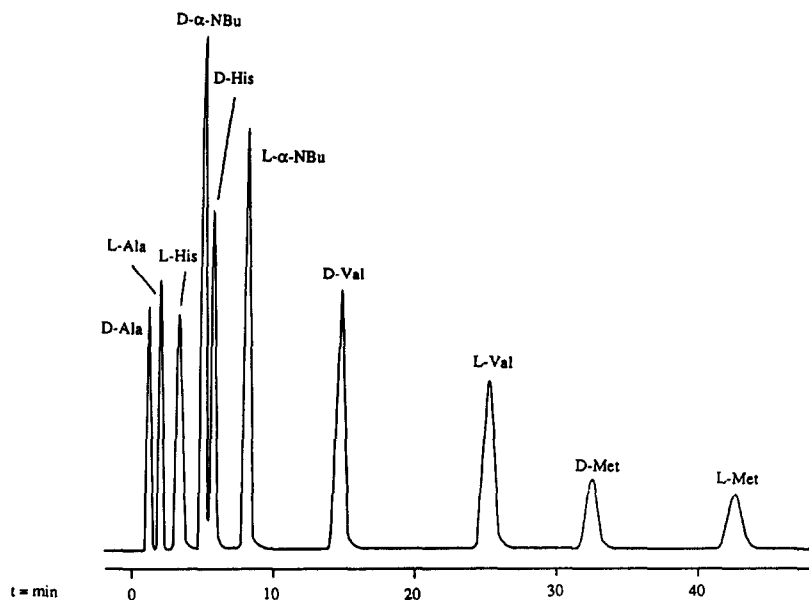
Fig. 11. Isocratic separation of a mixture of D,L-amino acids with L-MePhe-A–Cu(II). Conditions: 2 m*M* L-MePhe-A, 1 m*M* copper(II) acetate dissolved in water; pH 6; column, Spherisorb ODS-2 (3 μm, 15 × 0.46 cm I.D.); room temperature; flow-rate, 0.5 ml/min; fluorescence detection (postcolumn derivatization with OPA).

ture of amino acids with fairly good chemoselectivity and high enantioselectivity (Fig. 11). However, we have not developed the method further, because in the meantime we envisaged an alternative two-dimensional system that allowed the chiral separation of one amino acid at a time (single amino acid analysis) [36].

Mechanism of chiral discrimination

Generally, chiral discrimination is ascribed to a reaction of ligand exchange between the initial copper complex and the enantiomers with formation of diastereomeric ternary complexes of different stabilities and/or different affinities for the column.

Evidence was obtained for the first time that a ligand-exchange mechanism is indeed occurring in the chromatographic system with copper(II) complexes of these bidentate ligands (L:Cu = 2:1). The experiment was performed with L-Trp-A, which is fluorescent. As copper(II) is a fluorescence quencher, in the binary complex bis(L-tryptophanamidato)copper(II), fluorescence was almost completely quenched (only 10% of the initial fluorescence was retained).

When using this complex as eluent and a fluorescence detector, a unique signal was observed with the same retention time for all D,L-amino acids, corresponding to free L-Trp-A. The mixed copper complexes, being non-fluorescent, were not detectable under the conditions used (Fig. 12). Indeed, fluorescence was induced by displacement of one Trp-A ligand from the initial bis(amino acid amidato) complex, according to the equilibrium

$$[Cu(Trp\text{-}A)_2] + AA \rightleftharpoons [Cu(Trp\text{-}A)\cdot(AA)]$$
$$+ \, Trp\text{-}A$$
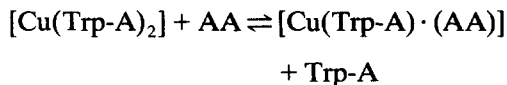
Moreover, when a UV detector was used at 254 nm, three positive signals were obtained, one corresponding to L-Trp-A and the other two to the diastereomeric ternary complexes. Absorption in this wavelength range is due to a charge-transfer band characteristic of these copper(II) complexes. When the UV detector was set at 280 nm, the absorption wavelength of the indole moiety of tryptophanamide, the signal corresponding to free L-Trp-A was present, whereas the two diastereomeric mixed complex-
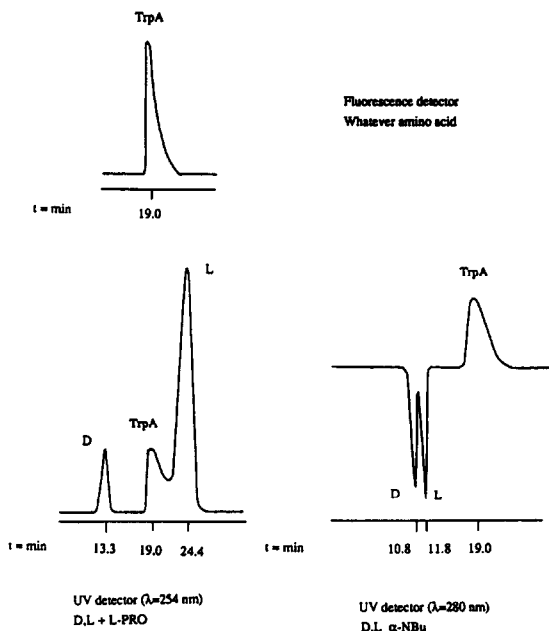
Fig. 12. Ligand-exchange experiment with L-Trp-A–Cu(II) system. Conditions: 0.4 mM L-Trp-A, 0.2 mM copper(II) acetate dissolved in water; pH 5.8; column, Spherisorb ODS-2 (3 μm, 15 × 0.46 cm I.D.); room temperature; flow-rate, 1 ml/min.

es gave rise to two negative peaks as they have a lower absorbance than the initial bis-tryptophanamidato complex, being deprived of a tryptophanamide moiety. The experiment unequivocally shows that one of the two ligands is displaced from the original binary complex to give rise to mixed species.

Whether ligand exchange occurs on the stationary phase or in the aqueous phase depends on the structural features of the selector [37]. With lipophilic selectors the chiral initial complex is completely adsorbed on the stationary phase and the recognition process occurs entirely in this phase [38,39]. With hydrophilic selectors, such as cyclodextrins, chiral discrimination is assumed to occur mainly in the mobile phase [40]. In a more general case, complex formation equilibria are relevant in both the mobile and stationary phases and partitioning of all species between the two phases must be taken into account to explain enantiomeric discrimination.

In the case of amino acidato–copper(II) com-

plexes as chiral additives [41], the three fundamental interactions accounting for the separation were assumed to be the coordination of the α-carboxy and the α-amino groups of the solute to the copper(II) ion and the hydrophobic interactions between the side-chains of the selector and the solute, which determine the stability of the ternary complexes in aqueous solution. According to our results with amino acid amidato–copper(II) complexes, the enantioselectivity observed in the chromatographic system is not accounted for by the formation equilibria of the diastereomeric complexes in solution [42], but rather by a complex series of equilibria in both aqueous and organic (stationary) phases. Whether one predominates leading to enantiomeric discrimination depends on the structural features of the initial complex and to the partitioning of the mixed complexes between the two phases. In agreement with Davankov et al. [43], the solvophobic interactions between the nonpolar side-chains of the selector and the solute with the stationary phase of the column appear to be fundamental.

As for the structures of the ternary complexes, we must consider that, in the pH range considered, three species are present: $CuLH^+_{-1}$, $CuL_2H^+_{-1}$ and CuL_2H_{-2}. However, if a ligand-exchange mechanism is occurring, the three species give rise to the same diastereomeric ternary complexes: Cu(II)(LH$_{-1}$)(L-amino acid) and Cu(II)(LH$_{-1}$)(D-amino acid). We assume that the diastereomeric complexes, formed by amino acid amides and amino acids, are *trans*, in agreement with the crystallographic data for the binary complexes of bis(amino acid amidato)–[31,32] and bis(amino acidato)–copper(II) complexes [44]. Accordingly, in the ternary L,L-
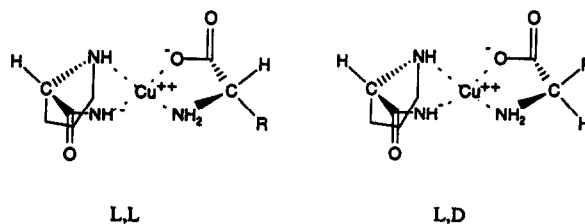

Fig. 13. Structures of the mixed ternary complexes L-Pro-A–Cu(II)–amino acid.
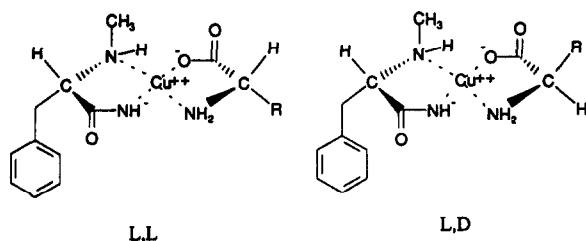
Fig. 14. Structures of the mixed ternary complexes L-MePhe-A–Cu(II)–AA.

species, the side-chains of both the selector and the enantiomer are situated on the same side of the copper coordination plane, thus allowing a strong interaction with the column when R is non-polar, and a less favourable interaction when R is hydrophilic; the opposite occurs with the D-enantiomers (Figs. 13 and 14). This interpretation accounts for the reversed elution order observed for polar and non-polar amino acids.

In fact, non-polar amino acids can interact strongly with the stationary phase of the column and are well separated by all ligands (in particular by more hindered ligands such as Me_2Phe-A and Pro-A). Polar amino acids were separated only by the more hydrophobic ligands (Phe-A and MePhe-A), whereas MeVal-A, Me_2Val-A and Pro-A do not ensure sufficient solvophobic interactions with the stationary phase. With Me_2Phe-A, polar amino acids were eluted too fast from the column, probably on account of the low stability of the complexes, without separation.

The behaviour of each polar amino acid is worth examining. Lysine and arginine were not separated, probably because in the pH range considered the protonated side-chain inferred

too low a hydrophobicity ($\pi_{Lys} = -1.14$, $\pi_{Arg} = -0.90$) to the amino acid and to the mixed complexes, thus preventing enantioseparation. In contrast, histidine ($\pi_{His} = -0.40$) was very well separated, as imidazole may form a five-membered chelate ring with the copper(II) ion at the apical position.

The possibility of apical interaction is probably the reason for the different chromatographic behaviour of aspartic and glutamic acid. In fact, it was possible to obtain the enantioselective separation of the former whereas the latter was not resolved. Although the two charged amino acids have very close hydrophobic coefficients ($\pi_{Asp} = -1.05$, $\pi_{Glu} = -0.98$), only the former can form a five-membered chelate ring with copper(II) at the apical position.

For threonine ($\pi_{Thr} = 0.33$) it is probably the presence of the methyl group in the side-chain which accounts for the separation; in contrast, serine is too hydrophilic ($\pi_{Ser} = -0.08$) and unhindered to be discriminated.

The addition of sodium acetate increases the ionic strength of the mobile phase and favours ionization of both the selector and the amino acids and their solubility in the aqueous phase. Thus, the ligand-exchange equilibria preferentially occur in the mobile phase and are less stereoselective. The organic modifier induces the same effect on the enantioselectivity, decreasing the solvophobic interactions with the column and favouring partitioning of the initial chiral complex and of the mixed species towards the mobile phase.

Enantioselectivity increases with increasing concentration of the initial complex up to a certain value, then it decreases at higher concen-
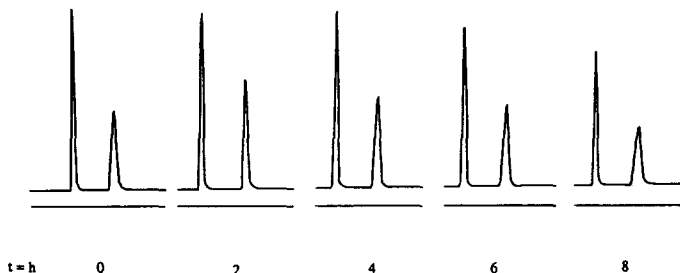

Fig. 15. Enantiomeric separation of D,L-α-aminobutyric acid with L-MePhe-A–Cu(II) adsorbed on the stationary phase of the C_{18} column. Conditions: 2 mM L-MePhe-A, 1 mM copper(II) acetate dissolved in water at pH 7 adsorbed on the column; eluent, water at pH 7; room temperature; column, Spherisorb ODS-2 (3 μm, 15 × 0.46 cm I.D.); flow-rate, 1 ml/min.

tration, suggesting that chiral discrimination mainly occurs on the saturated stationary phase. Accordingly, the lipophilic MePhe-A easily saturates the column at a 1 mM concentration, whereas a higher concentration is needed for Pro-A (4 mM). By further increasing the concentration of the initial complex in the mobile phase, the ligand-exchange reaction occurs also in solution, leading to faster elution of both enantiomers and to a decrease in α.

To confirm that the separation occurs on the stationary phase, we performed an experiment in which the complex with MePhe-A was previously adsorbed on the column and elution was carried out with water at pH 7. The column retained its efficiency and enantioselectivity for at least 8 h (Fig. 15). By adding a small amount of copper(II) acetate to the eluent, the enantioselectivity could be maintained for several weeks.

CONCLUSIONS

In conclusion, we have provided a novel, simple method for the enantiomeric separation of unmodified amino acids by HPLC. The selectors are easily available and give very good enantioselectivity factors. It has been shown unequivocally for the first time in HPLC that, in the case of bis(amino acid amidato) complexes, the mechanism proceeds via the exchange of one ligand from the binary complex. The system involves a series of equilibria of exchange in the aqueous solution, in the organic phase and between the two phases. The enantioselectivity due to complex formation in the mobile phase appears to be negligible with respect to the overall discrimination process, whereas the solvophobic interactions of the species with the column is of the utmost importance.

ACKNOWLEDGEMENTS

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