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BIS(L-AMINO ACID AMIDATO)COPPER(II) COMPLEXES AS CHIRAL ELUENTS IN THE ENANTIOMERIC SEPARATION OF D,L-DANSYLAMINO ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Copper(II) complexes of L-amino acylamides (Phe, Val, Tyr, Ala) when added to the eluent (water–acetonitrile) in reversed-phase high-performance liquid chromatography (C_{18}) are able to perform enantiomeric separation of dansylamino acids. The lipophilicity and bulk of the ligand greatly affect the stereoselectivity and the elution order of the enantiomers. The type and concentration of the copper complexes, pH and eluent polarity were examined in order to get some insights into the separation mechanism. This may be consistent with a ligand-exchange mechanism, probably occurring on the organic phase of the column, where the enantioselective complex is adsorbed. Mixtures of D,L-dansylamino acids were well separated by isocratic and gradient elution.

INTRODUCTION

Copper(II) complexes of L-amino acids[1–7] have been used as additives to the mobile phase for the chiral resolution of free or derivatized amino acids in reversed-phase high-performance liquid chromatography (HPLC). Other amino acid derivatives complexed to copper(II) have also been used, *i.e.*, L-hystidine methyl ester[8], N-tosyl-L-phenylalanine[9] and L-phenylglycine[10], N,N-dipropyl-L-alanine[11], aspartame and its methyl ester[12]. With alkylamides of the amino acids, L-aspartyl[13–15] and L-prolyl[16], respectively complexed to copper(II) and nickel(II), the enantioselectivity was found to be dependent on the alkyl chain length of the resolving agent.

It is generally assumed[17] that, by analogy with resins incorporating L-amino acids, in the case of the chiral eluent the mechanism of chiral recognition proceeds via ligand exchange between the initial copper(II) complex and the incoming D- and L-amino acids to be resolved, leading to the formation of diastereomeric ternary complexes of different stabilities and/or affinities for the column.

More recently, Weinstein and Leiserowitz[18], on the basis of crystallographic studies, proposed an alternative mechanism involving coordination at the apical position of the initial copper complex and/or an outer sphere interaction.

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In a general scheme aimed at studying the mechanism of chiral recognition, we have recently[19] proposed as a model diamino-diamido type ligands which give rise to tetradentate copper(II) complexes of different structures and stabilities, which should undergo a slower rate of decomplexation and be more liable to apical or outer-sphere interaction with the enantiomers, rather than a simultaneous dechelation of two binding sites, as in the "classical" ligand-exchange mechanism. The chromatographic results reported in the preceding paper[20] are consistent with such an hypothesis.

It was opportune at this point to study the stereoselectivity of the copper(II) complexes of simple L-amino acylamides and to compare the results obtained with those for the corresponding bridged complexes. In this paper we report the separation of D- and L-dansyl (Dns) amino acids on a reversed-phase column (C_{18}) using mobile phases containing copper(II) complexes of L-phenylalanyl-, L-tyrosyl-, L-valyl- and L-alanylamides. Special attention has been paid to the structural features of the initial complexes, to their relative stabilities and affinities for the column. Other parameters, *i.e.*, pH, eluent polarity, concentration and ligand-to-metal ratios have been examined. On the basis of the results obtained and of the relative elution orders, the mechanism of enantiomeric separation is discussed.

Moreover, practical aspects of the method are considered and it is shown that, with unsophisticated and currently available reagents such as L-amino acylamides, it is possible to achieve very good enantiomeric separations of amino acid mixtures both by isocratic and gradient procedures.

EXPERIMENTAL

Equipment

Chromatographic analyses were performed on a Waters Model 440 chromatograph equipped with a Model 420 fluorescence detector and a Dani recorder. The gradient system was obtained by means of a Waters automated gradient apparatus. A C_{18} Novapak (4 μm, 15 cm $\times$ 0.4 cm) column was used.

Reagents

D,L- and L-Dns-amino acids and L-amino acylamides were obtained from Sigma. Acetonitrile (LC-grade) and copper acetate (RPE-ACS grade) were obtained from Carlo Erba (Milan, Italy). Water twice distilled in our laboratory was used.

Mobile phase preparation

The mobile phase was prepared by dissolving the appropriate amount of copper acetate and of the ligand AA-A in a 0.3 *M* aqueous solution of sodium acetate. Acetonitrile was added to the desired percentage. The pH was adjusted with concentrated potassium hydroxide to the required value. The eluent was filtered and degassed under reduced pressure. In order to equilibrate the system and get it ready for analysis, the mobile phase was allowed to flow through the column for about 20 min at a flow-rate of 0.5 ml/min.

Gradient conditions

Two aqueous solutions containing 4 m*M* ligand, 2 m*M* copper and 0.3 *M*

sodium acetate at different percentages of acetonitrile and at the same pH (7.3) were prepared. The automated mixing was performed via a Waters automated gradient apparatus according to the following program:

t (min) *For* L-*Val-A/Cu(II)*	*Buffer A (20% acetonitrile) (%)*	*Buffer B (28% acetonitrile) (%)*
0	100	0
20	80	20
50	20	80
70	0	100

For L-*Phe-A/Cu(II)*	*Buffer A (24% acetonitrile) (%)*	*Buffer B (31% acetonitrile) (%)*
0	100	0
15	80	20
50	10	90
70	0	100

RESULTS AND DISCUSSION

Structures of the initial copper complexes: pH effect

L-Amino acylamides (phenylalanyl, tyrosyl, valyl and alanyl) and copper acetate were added to the mobile phase (water–acetonitrile) at various pH and enantiomeric separation of Dns-amino acids in HPLC (reversed-phase) was performed on a C_{18}-Novapak column. On going from pH 6.8 to 8.4, in the case of apolar Dns-amino acids, the selectivity coefficient, α, increases with pH, whereas for polar Dns-amino acids it reaches a maximum at around pH 7.5 and then decreases (see Fig. 1, for ligand L-Phe-A).

Now, from data available in the literature for glycinamide[21] and from our own

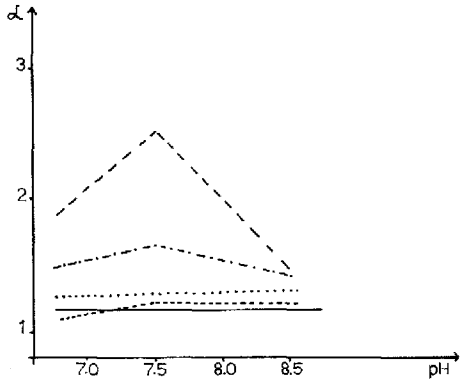

Fig. 1. Variation of the enantioselectivity coefficient, α, as a function of pH with L-Phe-A/Cu(II). Conditions: 4 m*M* L-Phe-A; 2 m*M* copper acetate; 0.3 *M* sodium acetate; water–acetonitrile (72:28); column, 15 cm × 0.4 cm, 0.4 μm C_{18} Novapak. $\alpha = k'_L/k'_D$ for Glu, Asp and Ser; k'_D/k'_L for Phe and Trp. – – –, Glu; – · –, Asp; ———, Ser; · · · · Phe; ---, Trp.

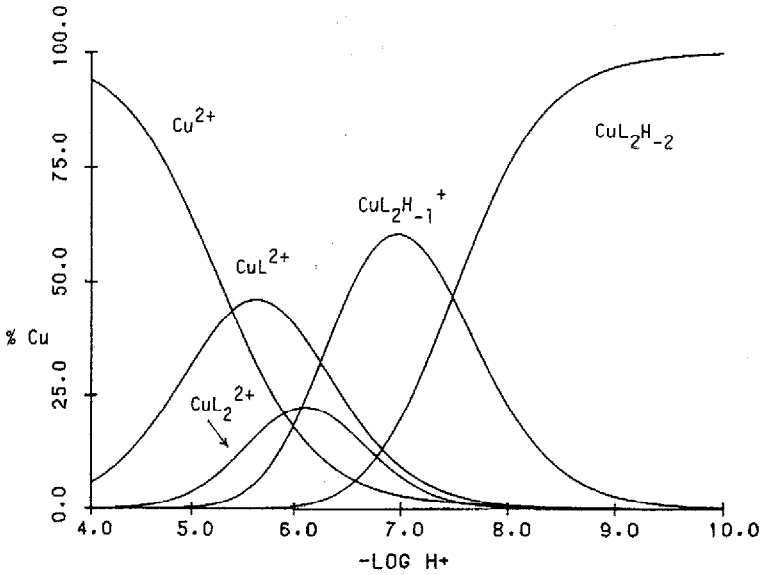

Fig. 2. Species distribution for the L-Phe-A/Cu(II) system as a function of pH, obtained by potentiometric titration.

data for L-Phe-A, L-Tyr-A, L-Val-A, L-Ala-A[22,23], it appears that in the pH range considered there are two copper complexes of the type $CuL_2H^{\pm}_1$ and CuL_2H_{-2} (Figs. 2 and 3) which may be responsible for the enantiomeric separation. However, it is not possible to establish here which complex is more enantioselective because other factors must be considered, such as the deprotonation of the sulphonamidic nitrogen of the Dns-amino acids [pK_a = 8.0 in the presence of Cu(II)][24]. For polar amino acids, which have a potentially chelating and/or charged side-chain, it is not necessary to reach basic pH in order to obtain the enantioselective interaction, whereas the deprotonation of the sulphonamide is more effective for apolar amino acids. In any case, it is remarkable that with these systems good enantiomeric separations may be obtained even at neutral pH (Figs. 4 and 5), whereas with other ligands it is necessary to use basic pH[25].

The species CuL_2H_{-2} was definitely shown to be enantioselective in the case of Phe-A [$Cu(Phe\text{-}A)_2H_{-2}$], which gave purple crystals suitable for X-ray determi-

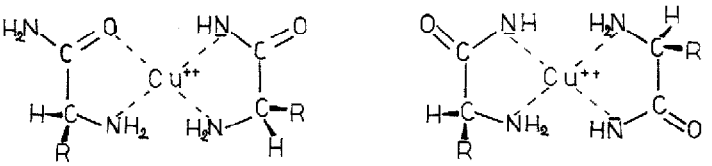

Fig. 3. Structures of copper complexes. (a) $CuL_2H^{\pm}_1$; (b) CuL_2H_{-2}.

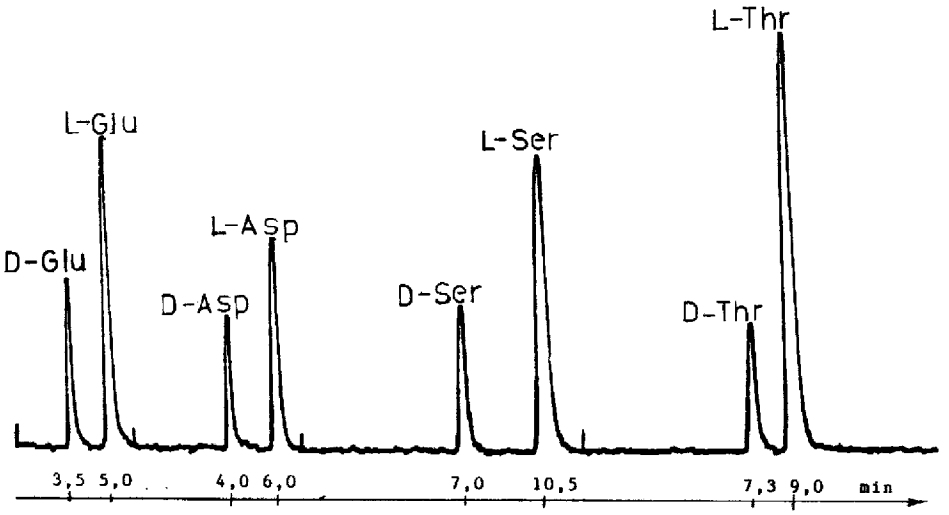

Fig. 4. Enantiomeric separation of Dns-amino acids by L-Phe-A/Cu(II). Conditions: 4 m*M* L-Phe-A; 2 m*M* copper acetate; 0.3 *M* sodium acetate; water–acetonitrile (72:28), pH 7.5; column, C_{18} Novapak.

nation[26]. When added to the solvent water–acetonitrile this complex facilitated resolution.

Lipophilicity and bulk of the ligand

The amino acylamides have different lipophilicities which can be estimated by the Hansch coefficient[27] [log *P*(Ala-A) = −2.23, log *P*(Val-A) = −1.43, log *P*(Tyr-A) = −0.71, log *P*(Phe-A) = −0.04] and different bulk. From Table I it is evident that the lipophilicity and the bulk of the amino acylamide side-chain determine the discrimination of the two enantiomers. Both Phe-A/Cu(II) and Val-A/-

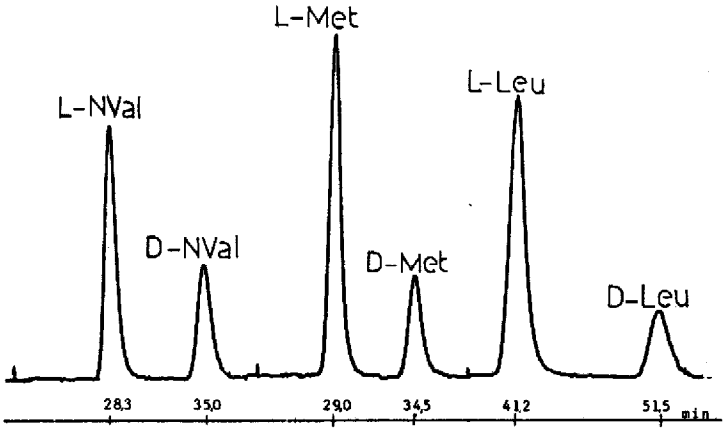

Fig. 5. Enantiomeric separation of Dns-amino acids by L-Val-A/Cu(II). Conditions: 4 m*M* L-Val-A; 2 m*M* copper acetate; 0.3 *M* sodium acetate; water–acetonitrile (77:23), pH 7; column, C_{18} Novapak.

TABLE I

ENANTIOMERIC SEPARATION OF Dns-AMINO ACIDS BY L-AMINO ACYLAMIDES AA-A/Cu(II)

Conditions: 4 m*M* L-amino acylamide; 2 m*M* copper acetate; 0.3 *M* sodium acetate; pH 7.5; 25°C. Column: 15 cm × 0.4 cm, 4-μm C_{18} Novapak. Eluents: water–acetonitrile (a) 72:28; (b) 77:23; (c) 82:18; (d) 78:22. $\alpha = k'_L/k'_D$; αNBu = α-aminobutyric acid.

Dns-amino acid	*Phe-A (a)*			*Tyr-A (b)*			*Val-A (b)*			*Ala-A (c)*		
	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α
Glu	0.64	1.45	2.30	0.77	1.27	1.64	0.50	1.27	2.54	1.05	1.41	1.34
Asp	1.00	1.64	1.64	1.09	1.68	1.54	0.82	1.68	2.05	1.23	1.68	1.37
Ser	3.00	3.59	1.20	2.82	3.50	1.24	2.09	3.73	1.78	3.64	4.41	1.21
Thr	3.09	3.54	1.15	3.23	3.23	1.00	2.32	3.23	1.39	4.32	4.73	1.09
αNBu	7.64	7.09	0.93	7.77	6.36	0.86	7.64	7.09	0.93	11.95	11.95	1.00
Val	9.91	8.82	0.89	10.95	9.59	0.87	10.91	9.77	0.89	19.55	19.55	1.00
NVal	15.09	12.09	0.80	15.59	12.73	0.82	17.00	13.54	0.79	27.63	25.64	0.93
Met	14.91	12.36	0.83	14.14	11.68	0.83	14.50	13.05	0.90	24.73	23.64	0.96
Leu	22.73	18.00	0.79	26.40	21.86	0.83	29.14	22.73	0.78	12.82	12.82	1.00(d)
Ala	6.50	6.00	0.92	5.18	4.41	0.85	5.36	5.91	1.10	2.77	2.77	1.00(d)
Phe	27.00	20.27	0.75	27.95	21.14	0.76	31.64	24.45	0.77	13.50	12.32	0.91(d)
Trp	37.18	31.64	0.85	32.41	24.77	0.76	34.27	37.36	1.09	15.45	13.32	0.86(d)

TABLE II

ENANTIOMERIC SEPARATION OF Dns-AMINO ACIDS BY Phe-A/Cu(II): INFLUENCE OF THE CONCENTRATION OF THE ADDITIVE

Conditions: column, 15 cm × 0.4 cm, 4-μm C_{18} Novapak; 0.3 *M* sodium acetate; water–acetonitrile (72:28), pH 7.5; V_0, 1.1 ml. $\alpha = k'_L/k'_D$.

Dns-amino acid	*L:Cu = 2 mM : 1 mM*			*L:Cu = 4 mM : 2 mM*			*L:Cu = 6 mM : 3 mM*		
	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α
Glu	0.36	0.82	2.25	0.64	1.45	2.30	0.54	0.91	1.67
Asp	0.73	1.27	1.75	1.00	1.64	1.64	0.64	1.00	1.57
Ser	2.18	3.09	1.42	3.00	3.59	1.20	1.02	2.91	1.28
Thr	2.36	2.82	1.19	3.09	3.54	1.15	2.64	2.91	1.10
αNBu	6.18	6.18	1.00	7.64	7.09	0.93	6.18	5.54	0.89
Val	6.54	6.27	0.96	9.91	8.82	0.89	8.82	7.54	0.86
NVal	10.36	8.64	0.83	15.09	12.09	0.80	11.91	9.18	0.77
Met	10.64	9.18	0.86	14.91	12.36	0.83	10.82	8.64	0.80
Leu	14.36	12.00	0.83	22.73	18.00	0.79	20.64	15.54	0.75
Phe	19.18	15.09	0.79	27.00	20.27	0.75	18.45	13.90	0.75
Trp	27.09	23.82	0.88	37.18	31.64	0.85	27.45	22.45	0.82

Cu(II) show good enantioselectivities, giving the best separation coefficients with resolution always >1.

A delicate balance of both factors is needed. When comparing two ligands with the same bulk, but different lipophilicities, such as Phe-A and Tyr-A, polar amino acids are better resolved by the former, apolar ones by the latter. The less bulky and lipophilic L-Ala-A/Cu(II) is inferior, although slightly better than the analogous bridged systems Ala-NN-2 and Ala-NN-3[20].

Concentration and ratio Cu(II)/L-amino acylamide

On account of the structure of the initial complexes, the ligand: Cu(II) ratio was maintained at 2:1, by varying the concentrations. In the concentration ranges $1 \leqslant \mathrm{Cu} \leqslant 3$ m*M*, $2 \leqslant \mathrm{L} \leqslant 6$ m*M* considered, polar amino acids are better resolved at low additive concentration whereas the apolar ones give better separations at higher concentration (Table II). This can be explained by assuming that the initial complex is adsorbed on the stationary phase, where it gives enantioseparation by ligand exchange with the enantiomers. Therefore, the best enantioselectivity is observed when the column is saturated with the initial complex. By further increasing the complex concentration in the eluent, the diastereomeric mixed complexes are formed in the mobile phase. Since both diastereomeric mixed complexes with polar amino acids are hydrophilic, they are less strongly retained by the column with consequent minor enantioselectivity. The mixed complexes with apolar amino acids, though less strongly retained, give different hydrophobic interactions with the column, thus producing higher separation coefficients. In any case, it is convenient to operate at L: Cu = 4 m*M* : 2 m*M*.

Eluent polarity

In order to have a good separation of the enantiomers, the initial complex must be allowed to establish lipophilic interactions with the organic phase coating the column. Therefore, the polarity of the mobile phase must be kept as high as possible, compatible with reasonable retention times. It was found to vary according to the lipophilicity of the ligand and the best conditions were: 28% acetonitrile for Phe-A, 23% for Val-A and Tyr-A, 18% for Ala-A.

The eluent contains also 0.3 *M* sodium acetate in order to buffer the solution and to reduce the retention times of Dns-amino acids[28].

Elution order

The elution order was checked for each amino acid by injecting a solution of the D,L-Dns-amino acid enriched with the L-enantiomer. For each amide it remains constant upon varying the pH. When comparing the elution order with the different amides/Cu(II) (Table I), we note that Phe-A, Tyr-A, Val-A and Ala-A show the same elution order: the D-enantiomer elutes before the L- for the polar amino acids and the L- before D- for the apolar ones (with the exception of Trp and Ala with Val-A). So, in general, all amide/Cu(II) complexes clearly distinguish between polar and apolar Dns-amino acids, which is not the case with the copper(II) complexes of the corresponding diamino-diamido ligands Phe-NN-2, Phe-NN-3, Val-NN-2, Val-NN-3 (which is always D-<L-)[20].

Analytical applications: resolution of a mixture of D,L-*Dns-amino acids*

Mixtures of several D,L-Dns-amino acids can be resolved under isocratic conditions. An example with L-Tyr-A is shown in Fig. 6.

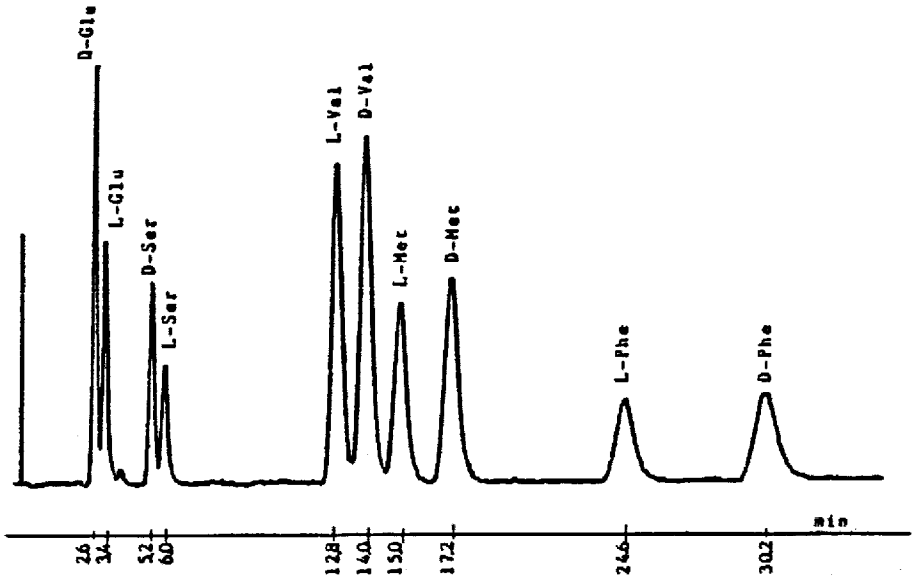

Fig. 6. Isocratic separation of a mixture of D,L-Dns-amino acids by L-Tyr-A/Cu(II). Conditions: 4 m*M* L-Tyr-A; 2 m*M* copper acetate; 0.3 *M* sodium acetate; water–acetonitrile (75:25), pH 7.5; column, C_{18} Novapak.

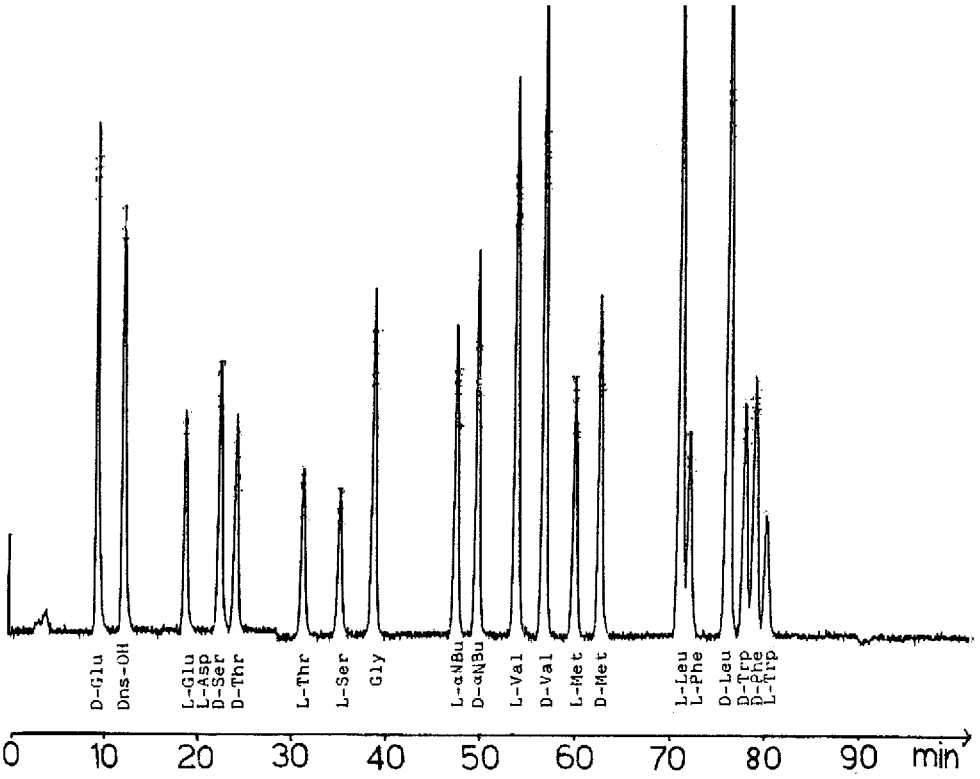

Fig. 7. Enantiomeric separation of a synthetic mixture of D,L-Dns-amino acids with L-Val-A/Cu(II). Conditions: 4 m*M* L-Val-A; 2 m*M* copper acetate; 0.3 *M* sodium acetate, pH 7; column, C_{18} Novapak; gradient conditions as in the Experimental.

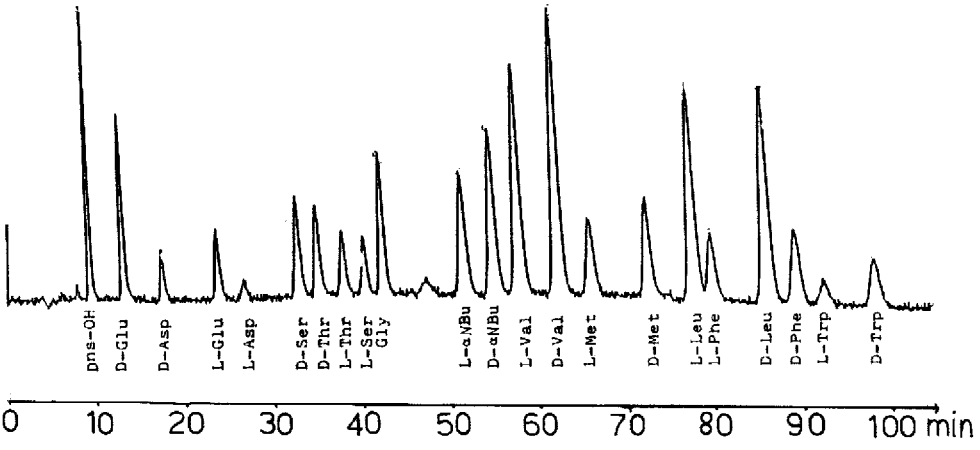

Fig. 8. Enantiomeric separation of a synthetic mixture of D,L-Dns-amino acids with L-Phe-A/Cu(II). Conditions: 4 m*M* L-Phe-A, 2 m*M* copper acetate. 0.3 *M* sodium acetate, pH 7.3; column, C_{18} Novapak; gradient conditions as in the Experimental.

Very good separations of complex mixtures can be achieved by means of a gradient, as shown for L-Val-A in Fig. 7 and for L-Phe in Fig. 8.

In our laboratories, by the combined use of these chromatographic systems, it has been possible to approach the determination of D-amino acids in several biological materials (cerebrospinal fluid[29], food[30], etc.).

Mechanism of resolution

On the basis of the considerations made in the case of the system AA-NN-*n*/-Cu(II)[20]: [best enantioselectivity displayed by the more lipophilic ligands (Table I), presaturation of the stationary phase with the initial complex (Table II), good performance on RP_{18}-TLC plates with no additive in the eluent[31]], also in the case of the system AA-A/Cu(II) it is reasonable to assume that the enantiomeric recognition occurs on the column where the initial complex is adsorbed.

Two complexes can be responsible for the enantiorecognition: $CuL_2H^{\pm}_1$ and CuL_2H_{-2} (Fig. 3). The negatively charged amino acids (Glu and Asp) are better resolved at neutral pH where the initial amide/Cu(II) complex is positively charged (Fig. 2); the opposite situation occurs with the apolar amino acids, which require a higher pH to become dianions (Fig. 1).

The enantioselective interactions with these systems must be different than those occurring with the system AA-NN-*n*/Cu(II), since a remarkably different elution order is observed in the two cases. Actually, with the present system AA-A/-Cu(II), it can be consistent with a ligand-exchange mechanism, where the ternary complex is formed by replacing one amide with the D- or L-enantiomer. This is reasonable because the initial binary amide/Cu(II) complexes have much more rapid complexation/decomplexation kinetics than that of the analogous tetradentate AA-NN-*n*/Cu(II) species.

Moreover, both complexes $CuL_2H^{\pm}_1$ and CuL_2H_{-2} would give rise to the same ternary complex D- or L-enantiomer/Cu/LH_{-1}, by ligand exchange of the weaker coordinated protonated amide, thus explaining the same elution order at the various pH values for each amide.

In order to explain the elution order observed with Phe-A, Val-A, Tyr-A, Ala-A, and in the absence of any experimental data on the complexation mode of Dns-amino acids in solution, it is reasonable to assume that at pH 7.5 the Dns-amino acid is coordinated to Cu(II) by the carboxylate group and the deprotonated sul-

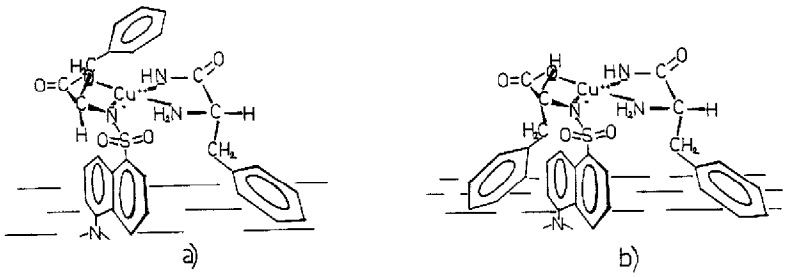

Fig. 9. Ternary complexes. (a) L-Phe-A/Cu/L-Dns-Phe; (b) L-Phe-A/Cu/D-Dns-Phe.

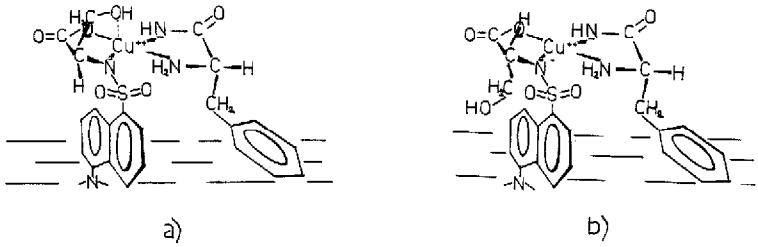

Fig. 10. Ternary complexes. (a) L-Phe-A/Cu/L-Dns-Ser; (b) L-Phe-A/Cu/D-Dns-Ser.

phonamidic nitrogen and that the latter is *trans* to the deprotonated amidic nitrogen of the ligand (Figs. 9 and 10).

Moreover, it seems feasible that the side-chain of the ligand and the naphthylamino group of the Dns-amino acid provide the main interaction with the organic phase of the column. Thus, the side-chain of the D-enantiomer gives an hydrophobic interaction with the column and is more strongly retained. On the contrary, the side-chain of the L-enantiomer is less accessible to the column and is less strongly retained. The mixed complexes of L-phenylalanyl amide and L-(a) and D-Dns-phenylalanine (b) are reported in Fig. 9. On the other hand, the D-enantiomer of the polar amino acids gives a repulsive interaction with the column and is eluted first. The L-enantiomer can eventually stabilize the ternary complex giving an apical interaction with the side-chain on Cu(II) and, thus be more strongly retained. The mixed complexes of L-phenylalanyl amide and L- (a) and D-Dns-serine (b) are shown in Fig. 10.

However, we do not exclude that a mechanism of enantioselectivity similar to that operating with the tetradentate complexes is involved here. In this case, the different elution order should be attributed to the different stereochemistries of the initial complexes (*trans* with AA-A and *cis* with AA-NN-*n*).

In conclusion, from the present chromatographic data, it appears that, a determining factor of the enantiomeric recognition is the lipophilic interaction of the mixed complexes with the column. It is feasible that a sequence of adsorption–desorption equilibria of the initial and mixed species occurs along the column leading to enantioseparation.

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