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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION OF D- AND L-Dns-AMINO ACIDS BY MIXED CHELATE COMPLEXATION

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SUMMARY

Separation of optical isomers of Dns derivatives of amino acids by reversed-phase high-performance liquid chromatography has been accomplished by adding a complex of an optically active amino acid to Cu(II) to the mobile phase. Cu(II) complexes of L-proline, L-arginine and L-histidine in the mobile phase yielded different degrees of separation. The concentrations of acetonitrile and the Cu(II) ligand as well as the pH all affect the separations. A chromatographic model is proposed that is based on the formation of ternary complexes by the D,L-Dns-amino acids and the chiral additive associated with the stationary phase. The separation selectivity appears to be based on different steric interactions between the alkyl side chains of the amino acids and the chiral additive.

INTRODUCTION

During the past decade attempts to resolve the optical isomers of amino acids by high-performance liquid chromatography (HPLC) have followed two general approaches. One used chiral ligands immobilized on the solid support to create a specific solute–sorbent interaction. In pioneering studies, Davankov and co-workers[1,2] resolved racemates by ligand-exchange chromatography using L-proline-bonded resins. The other technique employed optically active chelates in the mobile phase.

Dotsevi *et al.*[3] synthesized a chiral bi-naphthol crown ether stationary phase for complexing optically active amino acids in their protonated forms. More recently Pirkle and House[4] devised a chiral fluoroalcoholic phase bonded to silica gel for complexing 3,5-dinitrobenzoyl derivatives of D- and L-amino acids on the basis of charge transfer. Hara and Dobashi[5] developed L-valyl derivative bonded stationary phases for separating enantiomers of N-protected amino acid esters in normal-phase HPLC. Other variations, such as the use of polymers to which optically active valine[6] and leucine[7] were grafted have also been used.

Separation of the isomers on stationary phases bonded to a chiral ligand is particularly applicable to the isolation of racemates when recovery of the purified isomers is desired. Some of the phases have applicability that is limited to only a few

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amino acid pairs. Few of the required phases are commercially available perhaps because of the complexity of their synthesis and the difficulty of separating the optically active ligand from the synthetic mixture. These difficulties have made the use of chiral chelates as additives to the mobile phase a more attractive alternative.

Karger and co-workers[8,9] first reported the use of L-2-alkyl-4-octyldiethylenetriamine metal complexes in aqueous mobile phases for separating optically active Dns-amino acids. Hare and Gil-Av[10] used a proline–Cu(II) eluent for separating free D- and L-amino acids. Lam and Chow[11] used a proline–Cu(II) eluent to separate D- and L-Dns-amino acids with a C_{18} column. Gilon et al.[12], using metal aspartate eluents, demonstrated yet another approach to the resolution of free amino acids.

In this paper we describe a method for separating D- and L-Dns derivatives of 12 amino acids by adding Cu(II) complexes of proline and arginine. The use of the Dns derivative rather than the free amino acid permits the formation of the mixed complexes and their chromatographic behavior to be studied. The interplay between pH and the concentrations of acetonitrile and Cu(II) complex, all of which influence the retention characteristics and the separation of the isomers was studied.

EXPERIMENTAL

Instrumentation

The chromatograph was a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 601 LC equipped with a Rheodyne 7105 injection valve, a Model LC 650-10 fluorescence spectrophotometer and a Model 56 chart recorder. The analytical column for the proline system was a 25 × 0.26 cm C_{18} column, ODS-HC SIL-X-1, from Perkin-Elmer. The columns for the arginine and histidine studies were packed with Nucleosil 5 purchased from Chrompack (Whittier, CA, U.S.A.). The column was packed by the downward slurry technique.

Reagents

Acetonitrile distilled in glass was bought from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). D- and L-Dns-amino acids were bought from Sigma (St. Louis, MO, U.S.A.) and Pierce (Rockford, IL, U.S.A.). Some were also prepared by the procedure of Olson *et al.*[13].

Procedures

The fluorescence at 480 nm was monitored with excitation at 340 nm.

RESULTS

A mobile phase containing L-proline and Cu(II) in a 2:1 molar ratio resolved a number of Dns-amino acids (Figs. 1 and 2).

The separation of the amino acids was dependent on the acetonitrile concentration. With lower concentrations the solutes were retained longer and resolution of the isomers was improved (*cf.* Figs. 1 and 3) The selectivity was maximal at 20% acetonitrile (Table I). The selectivity (α) and capacity factors (k') changed markedly when the pH was changed from pH 5 to pH 7. At the higher pH the selectivity was

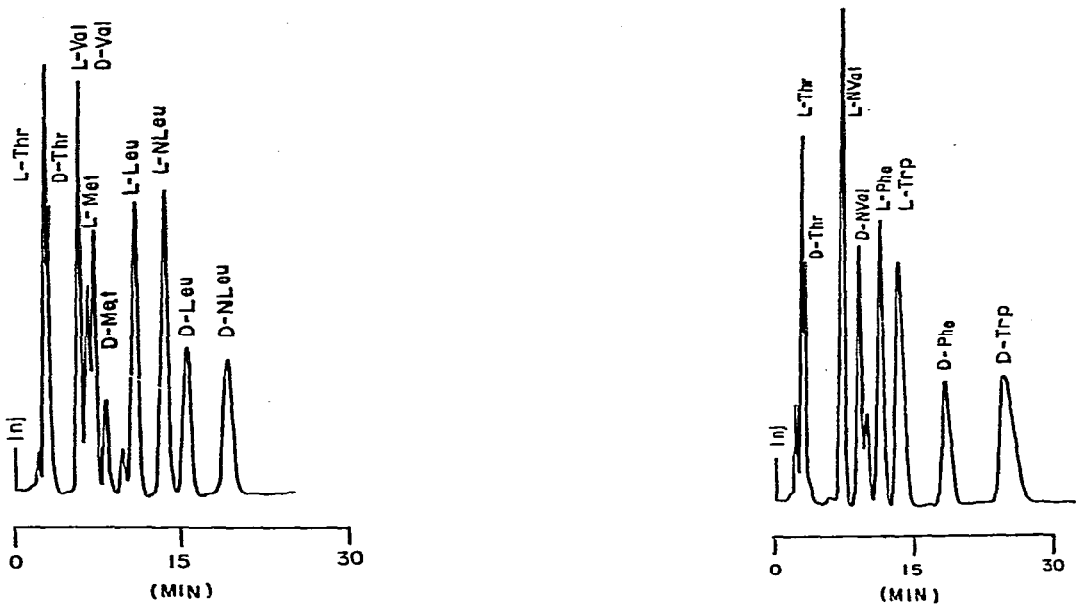


Fig. 1. Separation of D,L-Dns-amino acids with L-proline–Cu(II) eluent. Mobile phase: 20% acetonitrile in an aqueous solution containing $5 \cdot 10^{-3}$ *M* L-proline and ammonium acetate, and $2.5 \cdot 10^{-3}$ *M* $CuSO_4 \cdot 5H_2O$, pH 7.0. Flow-rate: 1.0 ml/min.

Fig. 2. Separation of D,L-Dns-amino acids with L-proline–Cu(II) eluent. Mobile phase: 20% acetonitrile in an aqueous solution containing $5 \cdot 10^{-3}$ *M* L-proline and ammonium acetate, and $2.5 \cdot 10^{-3}$ *M* $CuSO_4 \cdot 5H_2O$, pH 7.0. Flow-rate: 1.0 ml/min.

greater (Fig. 4). At pH 5 only the isomers of tryptophan were resolved even though the retention of the amino acids was greater than at higher pH (Fig. 5).

The concentration of the proline–copper complex also affected the separation. At 1 m*M* proline and 0.5 m*M* Cu(II) there was no separation of the smaller amino acid pairs and only slight separation of the larger. As the concentration was increased the k' increased up to a maximum at approximately 20 m*M* proline and 10 m*M* Cu(II). The resolution of enantiomers became constant and was maximal at that concentration (Fig. 6, Table II).

With L-arginine–Cu(II) added to the mobile phase, instead of proline–Cu(II), many amino acid pairs were separated (Fig. 7, Table III). The selectivity was in the reverse order to that with L-proline–Cu(II): the D-isomers eluted before the L-isomers.

Substituting histidine–Cu(II) complex for the proline complex permitted D- and L-aminobutyric acids and D- and L-alanine to be resolved (Fig. 8).

DISCUSSION

The simplest form of a mixed chelation is a ternary complex consisting of a metal ion and two non-identical ligands. Such complexes have been found in physiological fluids[14] and enzyme–metal-substrate complexes[15]. Mixed ligand–metal ion complexes of amino acids and peptides have been studied extensively.

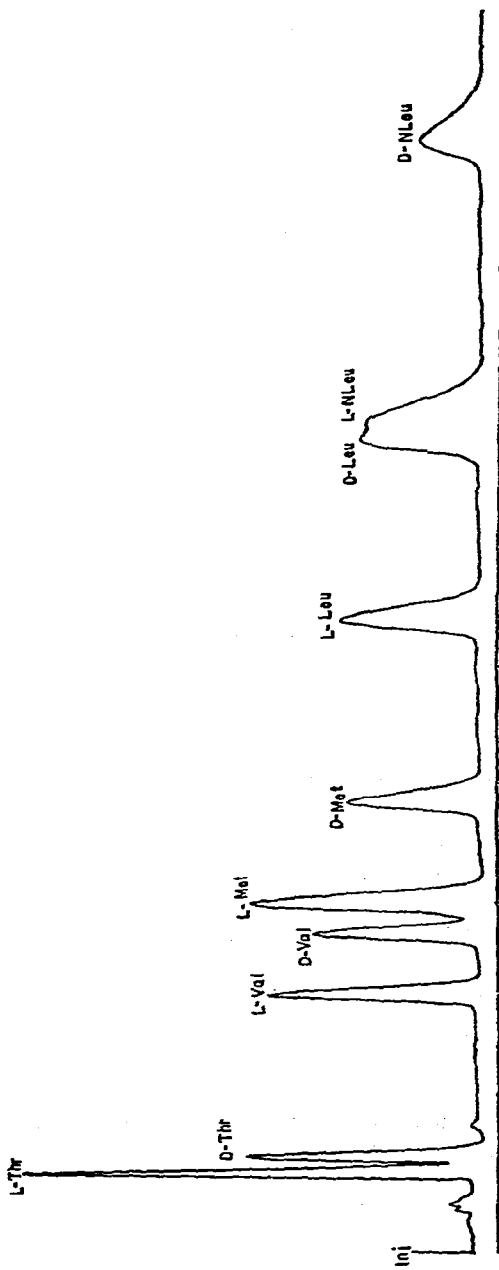


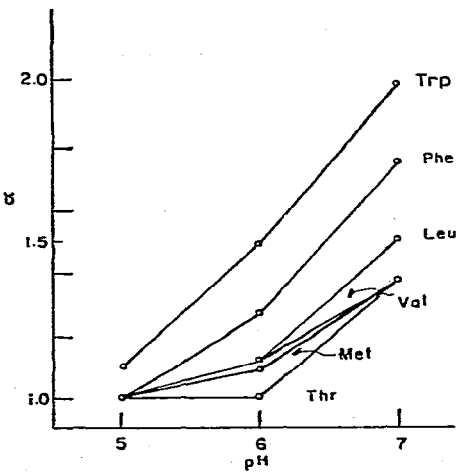
Fig. 3. Separation of D,L-Dns-amino acids with L-proline–Cu(II) eluent. Conditions as in Figs. 1 and 2, except the mobile phase acetonitrile concentration was changed to 15%.

TABLE I

CAPACITY RATIO (k') AND SELECTIVITY (α) AS A FUNCTION OF ACETONITRILE CONCENTRATION

Mobile phase: acetonitrile (percentage indicated in the table) in an aqueous buffer containing $5 \cdot 10^{-3}$ *M* L-proline and ammonium acetate, and $2.5 \cdot 10^{-3}$ *M* $CuSO_4 \cdot 5H_2O$; pH 7.

Solute	*Acetonitrile (%)*					
	25		*20*		*15*	
	k'	α	k'	α	k'	α
D-Thr	0.34	1.00	0.97	1.37	5.17	1.24
L-Thr	0.34		0.71		4.17	
D-Ser	0.35	1.00	0.79	1.00	3.83	1.00
L-Ser	0.35		0.79		3.83	
D-Met	1.57	1.15	4.71	1.37	22.93	1.27
L-Met	1.36		3.43		18.03	
D-Val	1.86	1.16	3.83	1.37	16.67	1.22
L-Val	1.60		2.79		13.67	
D-Leu	3.00	1.27	9.86	1.50	41.40	1.29
L-Leu	2.36		6.57		31.97	
D-N-Val	1.64	1.21	5.07	1.39	21.83	1.27
L-N-Val	1.35		3.64		17.17	
D-N-Leu	3.54	1.38	12.43	1.47	55.50	1.31
L-N-Leu	2.86		8.43		42.33	
D-Phe	4.21	1.39	11.64	1.75	71.83	1.77
L-Phe	3.03		6.64		40.67	
D-Trp	4.43	1.72	19.37	1.97	73.67	1.60
L-Trp	2.57		9.80		46.07	



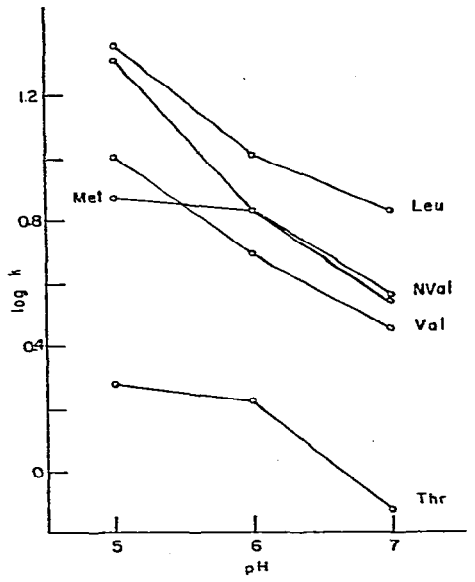


Fig. 4. Selectivity (α) as a function of pH. Mobile phase: 20% of acetonitrile in an aqueous buffer containing $5 \cdot 10^{-3}$ *M* L-proline and ammonium acetate, and $2.5 \cdot 10^{-3}$ *M* $CuSO_4 \cdot 5H_2O$, pH as given in the figure.

Fig. 5. Influence of pH on the retention of L-Dns-amino acids. Conditions as in Fig. 4.

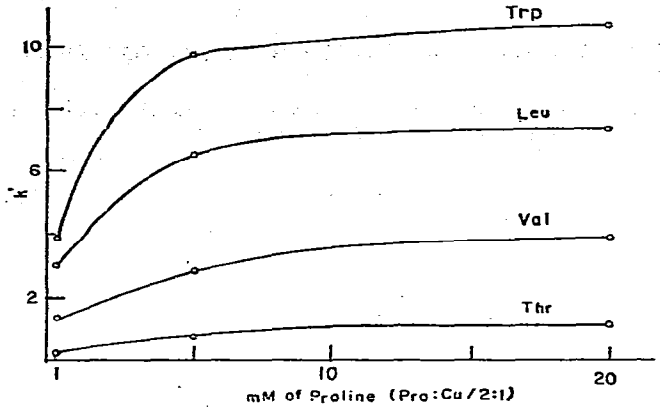


Fig. 6. Influence of L-proline–Cu(II) concentration on capacity ratio (k'). Conditions as in Table II.

In a solution containing a metal ion M(II) and an excess of anions of two different amino acids, Ax^- and Ay^-, the following equilibria can be postulated to exist (ignoring the charges and the D,L-forms):

$$M + 2\,Ax \rightleftharpoons M(Ax)_2$$
$$M + 2\,Ay \rightleftharpoons M(Ay)_2$$
$$M + Ax + Ay \rightleftharpoons M(Ax)(Ay)$$
$$M(Ax)_2 + M(Ay)_2 \rightleftharpoons 2M(Ax)\,(Ay)$$

The disproportionation constant K can be written as:

$$K = [M(Ax)\,(Ay)]^2/[M(Ax)_2]\,[M(Ay)_2]$$

Statistically there are two ways the mixed complex M(Ax) (Ay) can be formed but only one way the binary complexes $M(Ax)_2$ and $M(Ay)_2$ can be. The expected dis-

TABLE II

CAPACITY RATIO (k') AND SELECTIVITY (α) AS A FUNCTION OF L-PROLINE–Cu(II) CONCENTRATION

Mobile phase: 20% of acetonitrile in an aqueous buffer containing $5\cdot10^{-3}$ *M* ammonium acetate, pH 7. Cu(II)–L-proline concentration as given in the table.

Solutes	*$1\cdot10^{-3}$ M L-Pro–$5\cdot10^{-4}$ M Cu(II)*			*$5\cdot10^{-3}$ M L-Pro–$2.5\cdot10^{-3}$ M Cu(II)*			*$2\cdot10^{-2}$ M L-Pro–$1\cdot10^{-2}$ M Cu(II)*		
	k' (L)	*k' (D)*	*α*	*k' (L)*	*k' (D)*	*α*	*k' (L)*	*k' (D)*	*α*
Thr	0.18	0.18	1.00	0.71	0.97	1.37	1.00	1.33	1.33
Met	1.76	1.76	1.00	3.43	4.71	1.37	4.53	6.13	1.35
Val	1.35	1.35	1.00	2.79	3.83	1.37	3.87	5.59	1.44
N-Val	1.50	1.50	1.00	3.64	5.07	1.39	4.87	6.60	1.36
Leu	3.00	3.41	1.14	6.57	9.86	1.50	7.20	12.73	1.77
N-Leu	4.29	6.05	1.41	8.43	12.43	1.47	10.67	15.93	1.49
Phe	3.70	4.88	1.32	6.64	11.64	1.75	10.06	15.47	1.54
Trp	3.88	5.49	1.41	9.80	19.37	1.97	10.40	20.67	1.99

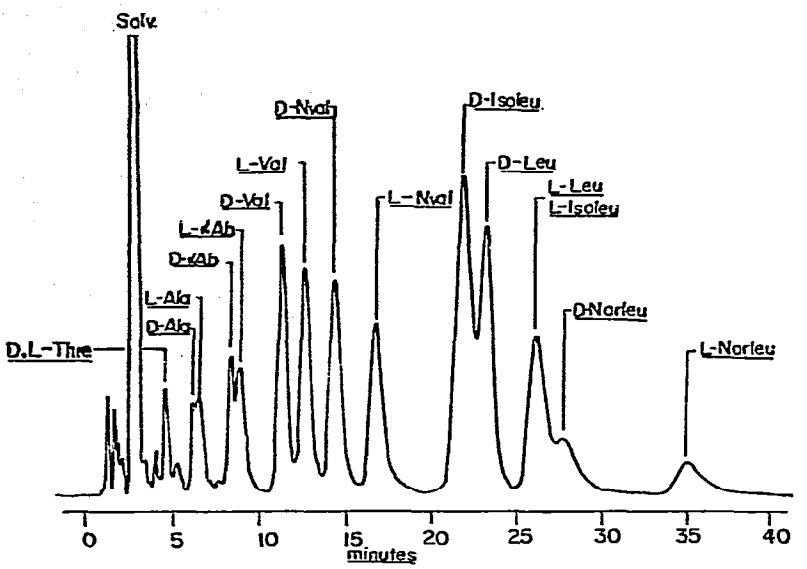


Fig. 7. Separation of D,L-Dns-amino acids with L-arginine–Cu(II) eluent. Mobile phase: 1:4 ratio of acetonitrile–aqueous solution containing $5 \cdot 10^{-3}$ *M* L-arginine, $2.5 \cdot 10^{-3}$ *M* $CuSO_4 \cdot 5H_2O$ and $2.5 \cdot 10^{-2}$ *M* ammonium acetate, pH 7.5. Flow-rate: 2.0 ml/min.

proportionation constant, *K* is 4. Moreover, mixed chelate formation should be strong if favorable electrostatic, steric and π-bonding effects are produced by complex formation[14,16].

In an aqueous mobile phase containing L-amino acid (L-A*x*) and Cu(II) in a 2:1 concentration ratio, the equilibria are:

$$\text{L-A}x + \text{Cu(II)} \rightleftharpoons (\text{L-A}x)\text{Cu(II)}$$
$$(\text{L-A}x)\text{Cu(II)} + \text{L-A}x \rightleftharpoons \text{Cu(II)}\,(\text{L-A}x)_2$$

TABLE III

CAPACITY RATIO (k') AND SELECTIVITY (α) OF D- AND L-Dns-AMINO ACIDS

Mobile phase: 1:4 ratio of acetonitrile–aqueous solution containing $5 \cdot 10^{-3}$ *M* L-arginine, $2.5 \cdot 10^{-3}$ *M* $CuSO_4 \cdot 5H_2O$, and $2.5 \cdot 10^{-2}$ *M* ammonium acetate, pH 7.5.

Solutes	*k' (D)*	*k' (L)*	*α*
Ser	3.00	3.00	1.00
Thr	3.00	3.00	1.00
Ala	4.17	4.42	1.05
α-Ab	5.67	6.16	1.09
Met	10.33	11.66	1.14
Val	8.33	9.50	1.14
N-Val	11.00	13.00	1.18
Leu	18.50	20.83	1.12
Iso-Leu	17.16	20.83	1.21
N-Leu	22.33	28.16	1.26
Phe	20.33	23.00	1.13
Trp	24.83	31.17	1.26

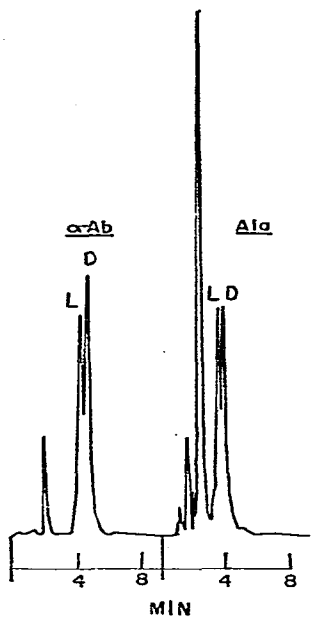


Fig. 8. Separation of D,L-Dns-α-aminobutyric acid and D,L-Dns-alanine with L-histidine–Cu(II) eluent. Mobile phase: 20% acetonitrile in an aqueous solution containing $5 \cdot 10^{-3}$ *M* L-histidine and ammonium acetate, and $2.5 \cdot 10^{-3}$ *M* $CuSO_4 \cdot 5H_2O$, pH 7.5. Flow-rate: 2.0 ml/min.

The species in the equilibrium responsible for the formation of the binary complex Cu(L-A*x*)$_2$ would be present in appreciable concentrations at neutral pH. Under conditions of reversed-phase chromatography the equilibrium would preclude significant concentrations of the charged species, (L-A*x*)Cu$^+$ and A*x*$^-$ in the aqueous phase. The more hydrophobic and neutral molecule Cu (L-A*x*)$_2$ would partition more in favour of the non-polar, C_{18} stationary phase (Fig. 9).

Since the steric effect is important in determining the disproportionation constant, stereoselectivity would be expected when mixed complexes of enantiomeric solutes, L-A*y* and D-A*y* are to form with L-A*x* partitioning in the stationary phase (Fig. 9).

Resolution of D- and L-isomers would be achieved when one of the ligands produces a stronger mixed complex and is able to disproportionate more of the binary complexes associated with the stationary phase. Such stereoselectivity would be reflected in the chromatographic process by k' and α. The presence of mixed complexes of proline and some amino acids with Cu(II) has been observed using circular dichroism[17].

The separation of the isomers was maximal in the proline system when the concentration of acetonitrile was 20%. This is attributable to the effect of polarity of the solvent on the acidity constants of both oxygen and nitrogen ligands. Sigel and McCormick[16] found that the changes in the dissociation of the complexes paralleled the changes in basicity. The dissociation of the nitrogen ligands increased only slightly as the polarity of the solvent decreased. The oxygen ligands dissociated less to about the same extent as the basicity with decreasing polarity of the solvent. With 25%

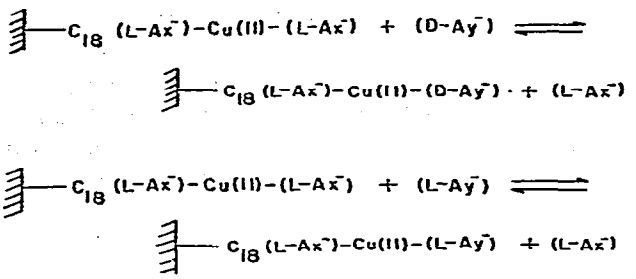


Fig. 9. model of partitioning of the D and L solutes on the stationary phase.

acetonitrile the selectivity between D- and L-forms was smaller than at 20% probably because the effect of hydrophobic interaction was greater. At 15% acetonitrile the more favorable partition coefficient is offset by the increased dissociation of the ligands. Threonine, which is not resolved at 25% acetonitrile, is resolved at 20% and below because of the greater effect of hydrophobic interaction at lower concentrations of acetonitrile.

The selectivity between D- and L-pairs depends on the alkyl substituent on the α-carbon of the amino acid (Table I, Fig. 4). The higher the carbon content and the bulkier the alkyl group, the larger is the selectivity factor, because of the interaction of the alkyl groups of the bis(amino acid)Cu(II) complex. The greatest selectivity is seen with phenylalanine and tryptophan which have the largest alkyl substituents.

For isomers with an equal number of carbon atoms, those pairs with a linear side-chain such as norvaline and norleucine are retained longer than those with a branched side-chain such as valine and leucine and are separated more. This behavior is probably due to the stronger spatial interaction of the straight chain isomers. The order of elution of L-isomer before the D-isomer suggests a more stable L-Pro–Cu(II)–D-amino acid complex[1,2]. This order of elution is the same as that obtained with proline-bonded phases, which tends to support the mechanism suggested here.

Bifunctional amino acids can add two protons. Since the metal and hydrogen ions compete for the amino acid ligands, the ability of the amino acid to bind Cu(II) decreases at lower pH and the selectivity decreases. At pH 5 only tryptophan isomers were resolved, presumably because of the large steric effect and the hydrophobicity of the molecule. At pH 7 there is good separation of the other enantiomers.

In addition to the stability of the mixed complexes, the separation of the stereoisomers is affected by hydrophobic interaction. The derivatives with the highest carbon content preferentially partition on the hydrocarbon stationary phase. As the pH is reduced from 7 to 5 the derivatives are increasingly retarded. At pH 5 the Dns derivatives may be less dissociated. This too would favor predominance of hydrophobic interaction. Methionine and threonine, with polar substituent groups, do not interact as strongly with the stationary phase as do the other amino acids. That probably explains the leveling of the k' between 5 and 6 for methionine and threonine (Fig. 5).

Increasing the concentration of Cu(II)–proline results in the formation of more binary complexes in the stationary phase and causes an increase in k' (Fig. 6, Table II). As the concentration is increased further the k' values level off, suggesting that the stationary phase is saturated. As the k' levels off the separation of individual amino

acid pairs becomes maximal, suggesting that the determining factor is then only the degree of dissociation of the complexes.

L-Arginine has been known to form ternary complexes with Cu(II) and either aspartic or glutamic acids[18]. It has not to our knowledge been used before for chromatographic separation in the way described here. The results were striking in that with L-arginine the D-isomers of the Dns-amino acids were eluted before the L-derivatives, the reverse of the order obtained with proline–Cu(II). The order of separation of the amino acids, however, was the same: the small and polar amino acids eluted earlier and the bulky, larger molecules later. Again the selectivity between D- and L-pairs was substantial when the amino acid had large alkyl substituents, probably because of the mechanism described previously.

In an attempt to explain some of the selectivity we constructed molecular models of the ternary amino acid complexes. The elution of the L- before the D-isomer in the proline system suggests a *trans* conformation of the bis(amino) acids around the Cu(II) ion (Fig. 10). The lesser stability of the L–L complex is apparently due to the interaction between the α substituent of the Dns-amino acid and the proline ring in the same plane. Recent ligand-exchange chromatography and potentiometric studies with proline and other amino acids support the concept of this molecular conformation[19].

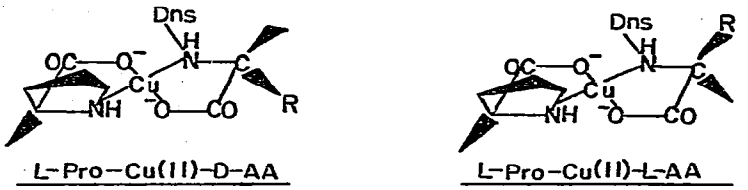


Fig. 10. Structure of L-proline mixed complexes.

The experimental results and the molecular models suggest that the *cis* conformation is favored for the arginine ternary complexes. This conformation would also allow electrostatic interaction of the positively charged guanido group of the arginine with the negatively charged carboxylate of the neighboring Dns-amino acid (Fig. 11). Electrostatic ligand–ligand interactions within the complex molecule have been responsible for the stereoselectivity in L-arginine–Cu(II)–D,L-glutamic acid, L-arginine–Cu(II)–D,L-aspartic acid[18] and L-arginine–Cu(II)–D,L-histidine[20] systems. The model would thus explain the early elution of the L-arginine–Cu(II)–D-amino acid complex which has both the alkyl group of the amino acid and arginine side-chain located in the same spatial coordinates.

Stimulated by the observations in potentiometric studies that stereospecific ternary complexes of histidine and other amino acids with Cu(II) can form[20], we tried a mobile phase containing L-histidine and Cu(II) in a 2:1 molar ratio. The D- and L-forms of α-aminobutyric acid and of alanine were separated. This work is in progress at the time of this writing.

To conclude, we have shown that resolution of D,L-Dns-amino acids can be accomplished by the addition of an optically active amino acid to the mobile phase, and separate the racemates in the form of ternary complexes. The implication of the

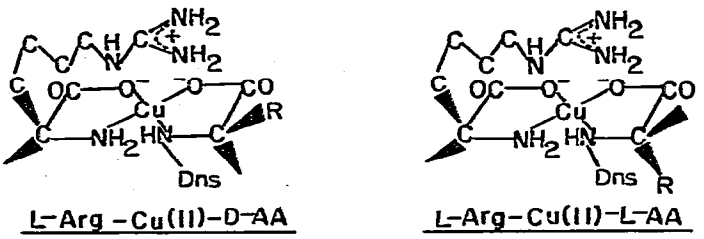


Fig. 11. Structure of L-arginine mixed complexes.

present study is above the interest of isomeric composition. It can be an easy approach to the study of labile mixed amino acid complexes.

ACKNOWLEDGEMENT

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