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REVERSED-PHASE LIQUID CHROMATOGRAPHIC RESOLUTION OF UNDERIVATIZED D,L-AMINO ACIDS USING CHIRAL ELUENTS

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SUMMARY

Resolution of underivatized amino acid enantiomers by reversed-phase liquid chromatography is described using chiral eluents containing the copper(II) complexes of the chiral chelates N-(*p*-toluenesulfonyl)-L-phenylalanine and N-(*p*-toluenesulfonyl)-D-phenylglycine. Resolution of the enantiomers of neutral, basic and acidic amino acids and their amides was accomplished on a octadecylsilyl bonded silica gel column. A chromatographic model is proposed that is based on dynamic ligand-exchange mechanism of D,L-amino acid with tosylated amino acid–copper(II) complex on the chemically bonded phase.

INTRODUCTION

High-performance liquid chromatographic (HPLC) resolution of amino acid enantiomers has been developed by using chiral derivatization reagents[1–3], chiral eluent[4] or chiral stationary phases[5]. Resolution utilizing a mobile phase containing a chiral additive is simple and furnishes the sensitive detection of enantiomeric amino acids by post-column derivatization with various reagents. We have recently reported[6] direct resolution methods for underivatized D,L-amino acids using a chiral mobile phase containing the copper(II) complex of N-(*p*-toluenesulfonyl)-L-phenylalanine (TosPhe) by reversed-phase HPLC on an *n*-octylsilyl (OS) bonded phase. This method provided excellent resolution of each of the amino acids, which were sensitively detected using *o*-phthalaldehyde (OPTA) reagent. D,L-Proline, which could not be detected by the resolution method using proline as the chiral additive[7], were detected by post-column derivatization with 7-chloro-4-nitrobenzofrazan (NBD-Cl) in this method[8].

In the present study, the copper(II) complex of N-(*p*-toluenesulfonyl)-L-phenylglycine (TosPhG) was found to be applicable to the resolution of the widest range of amino acids among the chiral additives so far reported.

MATERIALS AND METHODS

Amino acids and other reagents were purchased from Wako (Osaka, Japan) and Tokyo Chemical Indsutry (Tokyo, Japan). Chemically bonded octadecylsilyl

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(ODS) silica gel, Develosil ODS (particle size 5 μm) was obtained from Nomura Chemical (Seto-shi, Japan). OPTA was purchased from Funakoshi Pharmaceutical (Tokyo, Japan). Water and acetonitrile were distilled using glass apparatus before use. TosPhe and TosPhG were prepared as described by Theodoropoulos and Craig[9].

The mobile phase consisted of acetonitrile–water containing 1 m*M* TosPhe (or TosPhG) and 0.5 m*M* $CuSO_4 \cdot 5H_2O$. The pH of the mobile phase was adjusted to 6.0 with 5% aqueous sodium carbonate solution. The OPTA reagent was prepared by dissolving the following materials in 500 ml of the water and de-gassing: 17 g of boric acid, 15 g of potassium hydroxide, 2 ml of mercaptoethanol, 400 mg of OPTA dissolved in 5 ml of methanol and 1.5 g of EDTA2Na. Develosil ODS was packed in the stainless-steel column tube (10 cm × 4.0 mm I.D.) in our laboratory by the conventional slurry-packing technique. Other chromatographic apparatus and conditions were similar to those previously described[6].

RESULTS AND DISCUSSION

The concentrations of hydrogen ion and TosPhe–copper(II) complex or TosPhG–copper(II) complex in the mobile phase were set according to the basic approach described previously[6], except for ODS silica gel as the reversed-phase packing which showed better reproducibility of the retention time than the OS column. because the separation of amino acids was dependent on the acetonitrile concentration in the mobile phase, the pertinent concentration was chosen for each amino acid enantiomer.

Several methods[10–12] have been reported for the resolution of enantiomeric amino acids using chiral additives such as L-proline–copper(II)[4,7]. However, no conventional method has achieved the resolution of all of the neutral, basic and acidic amino acids together with their amides. Table I lists the capacity ratio (k'), separation factor (α), and difference in the free energies ($\Delta\Delta G^\circ = -RT \ln \alpha$) of D,L-amino acids resolved using TosPhe–copper(II) and TosPhG–copper(II) as chiral additives. All the amino acids tested, except glutamine, were resolved by the TosPhe–copper(II) system. Neutral amino acids were eluted in the order of L before D. On the other hand, basic and acidic amino acids and serine were eluted in the order D before L. D,L-Phenylglycine (D,L-PhG) showed the largest α value on the ODS column, suggesting that the stereoselectivity of the D,L-PhG–copper(II)–TosPhe complex is higher than those of other amino acids in the ligand-exchange reaction. This fact prompted us to develop TosPhG, which was expected to give better resolution than TosPhe owing to its sterically favored structure. In the present study, the α values of many amino acids were shown to increase by the use of TosPhG–copper(II) in place of TosPhe–copper(II) (Table I). The elution orders were reversed for all pairs of enantiomers compared with those observed for the TosPhe–copper(II) system. A typical resolution was observed for D,L-glutamine, which was not clearly separated with the TosPhe–copper(II) system as displayed in Fig. 1. Although the TosPhG–copper(II) system showed a slightly smaller separation factor in the limited case, it facilitated complete resolution of neutral, basic, and acidic amino acids and their amides.

Karger *et al.*[13] reported the resolution method for D,L-Dns-amino acids using L-prolyl-*n*-dodecylamide–nickel(II) as the chiral additive in reversed-phase HPLC. They have obtained data indicating that their chiral additive is distributed on the

TABLE I

CAPACITY RATIOS (k'), SEPARATION FACTORS (α), AND DIFFERENTIAL GIBBS FREE ENERGIES ($\Delta\Delta G^{\circ}$) OF D,L-AMINO ACIDS

Mobile phase, aqueous solution containing 1 mM TosPhe (or TosPhG), 0.5 mM $CuSO_4 \cdot 5H_2O$ and acetonitrile (percentage indicated in the table); pH 6.0; column, Develosil ODS-5 (10 cm × 4.0 mm I.D.)

Amino acid		*TosPhe*			*TosPhG*			*Acetonitrile (%)*
		k'	α	$\Delta\Delta G^{\circ}$	k'	α	$\Delta\Delta G^{\circ}$	
Serine	L	5.50	1.10	57	4.40	1.34	176	0
	D	5.00			5.90			
Aspartic acid	L	6.20	1.24	130	2.30	1.13	74	0
	D	5.00			2.60			
Asparagine	L	6.40	1.08	46	5.50	1.12	68	0
	D	5.90			6.14			
Glutamic acid	L	8.60	1.30	158	5.60	1.21	115	0
	D	6.60			6.80			
Glutamine	L	7.40	1.00	0	6.20	1.29	153	0
	D	7.40			8.00			
Alanine	L	5.50	1.31	163	7.60	1.10	57	0
	D	7.20			6.88			
Valine	L	7.80	1.77	344	14.10	1.34	176	10
	D	13.80			10.55			
Norvaline	L	2.70	1.48	236	4.40	1.73	330	15
	D	4.00			2.55			
Tyrosine	L	2.50	1.44	220	3.09	1.67	309	15
	D	3.60			1.85			
Phenylglycine	L	4.90	2.04	429	10.08	2.58	571	15
	D	10.00			3.90			
Isoleucine	L	4.80	1.71	323	8.12	2.08	441	15
	D	8.20			3.90			
Leucine	L	7.20	1.44	220	9.40	1.68	312	15
	D	10.40			5.60			
Norleucine	L	7.60	1.62	290	11.40	1.90	386	15
	D	12.30			6.00			
Phenylalanine	L	16.00	1.63	294	24.71	2.03	426	15
	D	26.00			12.19			
Tryptophan	L	22.00	1.64	298	34.20	1.93	396	15
	D	36.10			17.70			
Histidine	L	6.00	1.76	340	1.64	1.25	134	15
	D	3.40			2.05			
Lysine	L	11.60	1.09	52	6.05	1.31	163	15
	D	10.60			7.90			
Arginine	L	14.00	1.11	63	6.34	1.32	167	15
	D	12.60			8.38			

surface of an *n*-alkyl-bonded stationary phase and may act as an immobilized chiral phase. The copper(II) complexes of tosylated amino acid (TosAA) herein described may also exert a similar effect. The reversed-phase column, previously equilibrated by passing TosAA–copper(II) solution through it, retained the resolution power against amino acid enantiomers for more than 5 h when eluted with an aqueous solution containing only 0.5 mM copper sulfate, as depicted in Fig. 2. This indicates that

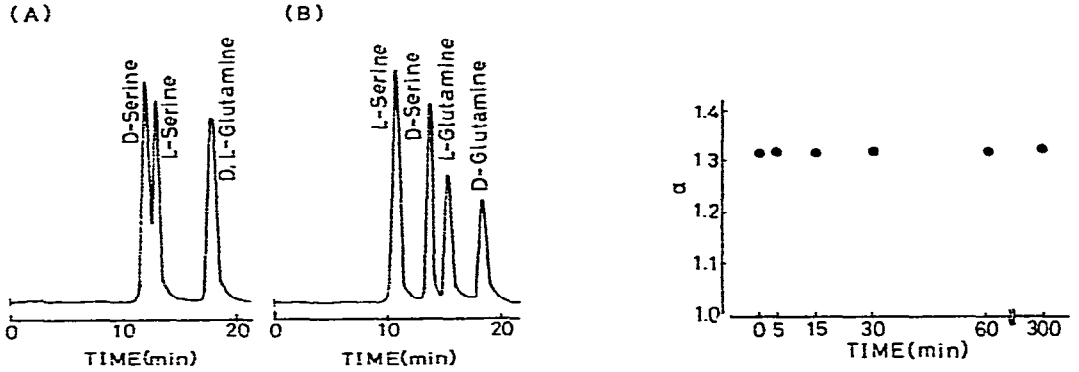

Fig. 1. Separation of D,L-glutamine and D,L-serine with (A) TosPhe–copper(II) and (B) TosPhG–copper(II) eluent system. Mobile phase, aqueous solution containing 1 m*M* TosPhe (or TosPhG) and 0.5 m*M* $CuSO_4 \cdot 5H_2O$; pH 6.0; *ca.* 0.25 nmol of each amino acid was injected.

Fig. 2. Change in α of D,L-alanine as a function of the time when eluted with an aqueous solution containing only 0.5 m*M* $CuSO_4 \cdot 5H_2O$. At zero time, the mobile phase is switched to one containing no chiral chelate (TosPhe).

TosPhe adheres to the surface of the ODS phase. Fig. 3 demonstrates the proposed resolution model based on the dynamic ligand-exchange mechanism for the stereoselective retention involving the labile immobilization of TosAA on the stationary phase. First the binary complex, $(TosAA)_2Cu$, in the mobile phase is adsorbed on the surface of the chemically bonded phase through hydrophobic interaction, and equilibrium is attained between the free and immobilized chelate complexes. Then the D- or L-amino acid injected into the column shifts in the column and triggers the ligand exchange with the immobilized chiral chelate. At this stage, an enantiomeric pair of amino acids may form ternary complexes of different conformation. The relationship between the resolution and the structure of the complex was explained in the preceding paper[6] as follows. The ternary complex of the D-amino acid may assume a *trans* conformation around the copper(II) ion and that of L-isomer a *cis* conformation. Molecular models support the fact that L-amino acid was eluted before the D-isomer because the *trans* isomer is thermodynamically more stable than the *cis* one[14]. However, the resolution may actually be influenced by the varying degree of hydrophobic interaction between the side-chain of the amino acids and the tosyl residue of the chiral additive and between the ternary complex of varying struc-

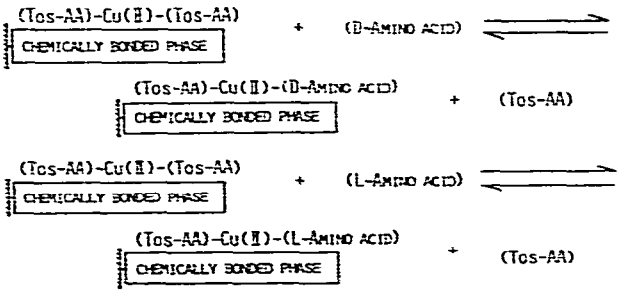

Fig. 3. Model of ligand-exchange mechanism of D,L-amino acid with TosAA–copper(II) complex on the stationary phase.

ture and the alkyl chain on the silica support. This may be the cause of the fact that some enantiomers are resolved in the order contrary to the above expectations, and that the separation factors of some amino acids in the TosPhG–copper(II) method are somewhat smaller than those in the TosPhe–copper(II) method.

The TosPhG–copper(II) system is expected to be suitable for the simultaneous resolution of amino acid enantiomers.

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