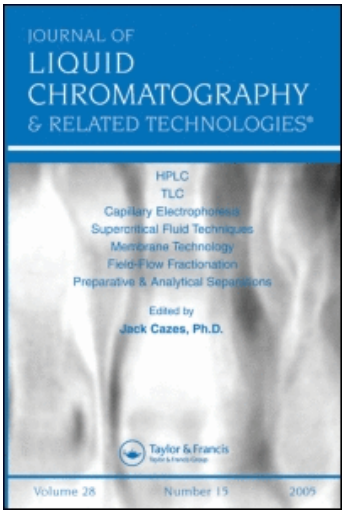

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Resolution of Optical Isomers as the Mixed Chelate Copper(III) Complexes by Reversed Phase Chromatography

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RESOLUTION OF OPTICAL ISOMERS AS THE MIXED CHELATE COPPER(II) COMPLEXES BY REVERSED PHASE CHROMATOGRAPHY

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ABSTRACT

Highly selective separation of D and L amino acids can be effected by reversed phase chromatography of mixed chelate complexes of the analyte acids with equimolar concentrations of Cu(II) and an optically active second amino acid in the mobile phase. The stabilities of the formed diastereomeric ternary complexes will determine the resolution of enantiomers. By this approach, amino acids were resolved as the dansyl and O-phthalaldehyde (OPA) derivatives and imino acids were separated without derivatization. Resolution of D and L dansyl amino acids was accomplished as the mixed complexes of Cu(II) with L-proline, L-arginine, L-histidine and L-histidine methyl ester as the second amino acids. Among the chiral ligands we studied, L-histidine methyl ester is unique in that it possesses both achiral selectivity for the dansyl amino acids and chiral selectivity for the respective D and L enantiomers. With a mobile phase gradient of acetonitrile in a buffer containing Cu(II) L-histidine methyl ester complex, we devised a stereoselective procedure for the analysis of D and L amino acid enantiomers, achieving the separation that the current amino acid analyzer failed. The mixed chelate metal

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complexation approach was recently extended to resolve OPA derivatives. The free amino acid was reacted with OPA in the presence of a chiral sulfhydryl reagent, N-acetyl-L-cysteine (NAC). HPLC of the derivatives was then performed on a reversed phase column, with a mobile phase containing Cu(II) L-proline, using fluorescence detection and resolved the optical isomers of the common primary amino acids. The same approach was also used to resolve compounds containing amine and sulfhydryl groups. The simultaneous detection and resolution of D and L imino acids is a more complicated problem. The reason is that the nitrogen of the imino acids must be derivatized in order to be detected whereas both the carboxyl and the imino groups must be free to allow complexation for chiral separation. A procedure for the resolution of D and L isomers of pipecolic acid and proline was devised by complexing the analytes with Cu(II)-L-aspartame and detecting the complexes at UV 235 nm. The urine concentration of D and L pipecolic acid was also measured this way for patients with disorders of lysine metabolism.

INTRODUCTION

Resolution of optical isomers of amino acids can be effected by complexation of the analyte acids with a chiral ligand and a metal ion introduced to the chromatographic system. Highly selective for the enantiomers, such a system can be implemented by two general approaches: one by immobilizing a chiral, metal coordinating ligand on the solid support to create a specific solute sorbent interaction, and the other by adding an optically active metal complex to the mobile phase to facilitate enantiomeric recognition.

An example of the first approach is the work of Davankov and Rogozhin who used a stationary phase consisting of L-proline bonded to resins on which Cu(II) metal ion was subsequently loaded. They pioneered the resolution of racemates of amino acids by ligand exchange chromatography (1,2).

In the last several years, interest in chiral separation stimulated the development of the second metal complexation

approach. In this, a non-polar, reversed phase column is usually used with a mobile phase containing a chiral metal complex. For example, Karger and co-workers (3,4) used L-2-alkyl-4-octyldiethylenetriamine complexes of zinc and other metals in the mobile phase to separate dansyl derivatives of amino acids. Hare and Gil-Av with Cu(II)-L-proline (5), Weinsteins et. al. with Cu(II)-di-N-propyl-L-alanine (6), and Nimur et. al. with (7) Cu(II) N-(p-toluene-sulfonyl)-L-phenyalanine resolved free D and L amino acids following by fluorescence detection after post column derivatization with OPA. Grushka et. al. (8,9), using Cu(II) complexes of L-aspartame and derivatives of aspartic acid also separated a number of D and L amino acids and detected the metal complexes at UV 235 nm.

Over the years, we have developed several stereoselective approaches to analyzing amino acids by HPLC. In the first we separated dansyl derivatives of amino acids on a reversed phase column as mixed chelate complexes with equimolar concentrations of Cu(II) and a second amino acid such as L-proline, L-arginine, L-histidine and L-histidine methyl ester in the mobile phases (10-16). Some chiral additives, such as L-histidine and L-proline, are more selective for optical isomers while others, such as L-histidine methyl ester and L-arginine, offer better separation of the different amino acids. Thus, L-histidine methyl ester was selected for the gradient separation of amino acids and their enantiomers.

In the second approach that was devised more recently, we separated stereoisomers of amino acids as derivatives formed with O-phthalaldehyde and the chiral sulfhydryl agent, N-acetyl-L-cysteine. All the primary amino acid pairs except those of histidine and cysteine, were separated rapidly on a reversed phase column with a mobile phase containing copper(II) L-proline. Our work with the amino acids indicated that the

complex formed between Cu(II) and the OPA-NAC derivatives resulted from binding of the copper at the carboxyl and N-acetyl amino group of N-acetyl-L-cysteine rather than at the coordination sites of the analyte amino acid. This observation suggested the use of this approach to resolve stereoisomers of primary amines and sulfhydryl compounds. The compounds selected for testing, D,L-phenylethylamine and D,L-normetanephrine as examples of interesting amino compounds, and N-acetyl-D,L penicillamine, which contains a sulfhydryl group, were all resolved (17).

Interest in assaying pipecolic acid and determining its optical isomers in urine had stimulated the development of the third approach for the imino acids. Imino acids were resolved on a reversed phase column as the Cu(II)-L-aspartame mixed complexes and which absorb at 235 nm were detected by a UV detector (18).

MIXED CHELATE COMPLEXATION

The chromatographic approach for the stereoselective resolution of amino acids bases on the mechanism on mixed chelate complexation of the analyte amino acids with the metal ion and a second amino acid added to the mobile phase. Mixed chelate metal complexes are commonly found in physiological systems as the mixed ligand-metal complexes of amino acids and peptides, and enzyme-metal-substrate complexes. The simplest form of a mixed chelation is a ternary complex consisting of a metal ion and two non-identical ligands. For the purpose of this discussion, we assume that the metal ion, M, is divalent and the ligands, Ax and Ay, are two amino acids. In solution the metal ion is in equilibrium with the two ligands as follows:

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

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

$$M + Ax + Ay \rightleftharpoons M(Ax)(Ay)$$

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

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 disproportionation constant is 4. Mixed chelate formation should be strong if favorable electrostatic, steric and pi-bonding effects are produced by complex formation (19).

The preferential formation of ternary metal complexes can be facilitated for enantioselective separations. Thus, in an aqueous mobile phase containing L-amino acid (L-Ax) and Cu(II) in a 2:1 concentration ratio, the equilibria are:

$$L\text{-}Ax + Cu(II) \rightleftharpoons (L\text{-}Ax)Cu(II)$$

$$(L\text{-}Ax)Cu(II) + L\text{-}Ax \rightleftharpoons Cu(II)(L\text{-}Ax)_2$$

At neutral pH, the binary complex $Cu(II)(L\text{-}Ax)_2$ is predominant. On the introduction of the enantiomers of an amino acid, an equilibria of the parent complexes and the diastereomeric complexes would be established:

$$\text{Silica-}C_{18}\ (L\text{-}Ax)Cu(II)(L\text{-}Ax) + (D\text{-}Ay) \rightleftharpoons$$

$$\text{Silica-}C_{18}\ (L\text{-}Ax)Cu(II)(D\text{-}Ay) + (L\text{-}Ax)$$

$$\text{Silica-}C_{18}\ (L\text{-}Ax)Cu(II)(L\text{-}Ax) + (L\text{-}Ay) \rightleftharpoons$$

$$\text{Silica-}C_{18}\ (L\text{-}Ax)Cu(II)(L\text{-}Ay) + (L\text{-}Ax)$$

The charged species and the metal ions would remain in the aqueous phase, while the more hydrophobic and neutral binary and ternary complexes would partition into the non-polar stationary phase. In the presence of Cu(II)(L-Ax)$_2$, the enantiomeric solutes disproportionate the chiral ligands to form two diastereomeric ternary complexes of different stability. Resolution of the enantiomers results on separation of these complexes by reversed phase chromatography.

STEREOSELECTIVE SEPARATION OF D AND L DANSYL AMINO ACIDS

Using mobile phases that contained chiral metal complexes of Cu(II) L-proline, L-arginine, L-histidine, and L-histidine methyl ester, we have separated D and L dansyl amino acids by the mixed chelation approach and the isomers detected by fluorescence. The selectivity between D- and L- pairs depends on the alkyl substituent on the α-carbon of the amino acid (Table 1). 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. With the exception of the L-histidine system, the greatest stereoselectivity is always observed with the isomeric pairs of phenylalanine and tryptophan which have the largest alkyl substituent.

The selectivity between the individual amino acids depends on the hydrophobicity of the metal complexes. The derivatives with the highest carbon content favorably partition on the hydrocarbon stationary phase. 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 with the stationary phase.

TABLE 1

Capacity Ratio (k') and Selectivity (α) of D- and L-Dns-Amino Acids for Four Chiral Eluents Containing 2.5 mM Cu(II) Complexes of the L Amino Acids Shown in the Headings.

	Proline			Histidine			Histidine methyl ester			Arginine		
	k'_L	k'_D	α	k'_L	k'_D	α	k'_D	k'_L	α	k'_D	k'_L	α
Ser	3.7	3.2	0.9	–	–	–	9.3	10.7	1.2	3.0	3.0	1.0
Thr	3.7	4.6	1.2	–	–	–	9.3	9.3	1.0	3.0	3.0	1.0
Ala	5.7	6.6	1.2	1.8	2.4	1.3	11.9	11.9	1.0	4.2	4.4	1.1
αAB	7.3	9.2	1.2	2.2	3.8	1.7	13.6	15.0	1.1	5.7	6.2	1.1
Val	11.4	15.0	1.3	3.2	6.4	2.0	17.3	20.1	1.2	8.3	9.5	1.1
Met	11.4	14.8	1.3	4.4	7.8	1.8	27.2	30.7	1.1	10.3	11.7	1.1
N-Val	14.6	19.0	1.3	3.8	9.0	2.4	23.3	27.6	1.2	11.0	13.0	1.2
I-Leu	—	—	—	7.2	15.4	2.1	40.4	44.7	1.1	17.2	20.8	1.2
Leu	23.9	32.6	1.4	7.2	16.0	2.2	33.0	43.9	1.3	18.5	20.8	1.1
N-Leu	32.6	45.7	1.4	9.4	22.2	2.4	40.4	58.4	1.5	22.3	28.2	1.3
Phe	32.6	52.8	1.6	9.0	9.0	1.0	60.4	77.9	1.3	20.3	23.0	1.1
Trp	41.2	71.2	1.7	7.4	7.4	1.0	93.3	121.8	1.3	24.8	31.2	1.3

Acetonitrile concentration was 15% for the proline and histidine systems and 20% for the histidine methyl ester and arginine systems.

The chromatographic system containing the Cu(II) complex of histidine methyl ester, which offered adequate chiral selectivity for the enantiomers and excellent achiral selectivity for the different amino acids, was developed into a stereoselective analysis system. Using a carefully controlled gradient of acetonitrile in a buffer containing Cu(II) L-histidine methyl ester, most of the amino acids except proline, hydroxylproline, alanine, threonine and cysteine were separated and their isomers resolved in the same analysis (Figure 1). The separations between various L isomers are superior in some instances to those by simple reversed phase chromatography. It has all the advantages of reversed phase chromatography with the extra dimension of separation via metal complexation. With this system, a procedure for the measurement of amino acids in the cerebrospinal fluid was devised. All the major amino acids were separated as the dansyl derivatives. As expected, most of them were in the L-form.

Of particular interest is the measurement of cerebrospinal fluids from patients with inflammatory diseases of the central nervous system because the possibility of change in amino acid composition and detection of D isomers from bacterial origin. Several patients with bacterial meningitis had easily distinguishable cerebrospinal fluid amino acid composition from patients with negative bacterial culture (Figures 2 and 3). The meningitis patients also had a greater number of peaks with retention time of D amino acids which were presumably synthesized by the infectious bacteria. However, more works are need to positively identify the unknown amino acids.

RESOLUTION OF D AND L AMINO ACIDS AS THE OPA DERIVATIVES

In a continuing effort to resolve enantiomers, we have extended the mixed chelation approach to the OPA derivatives of

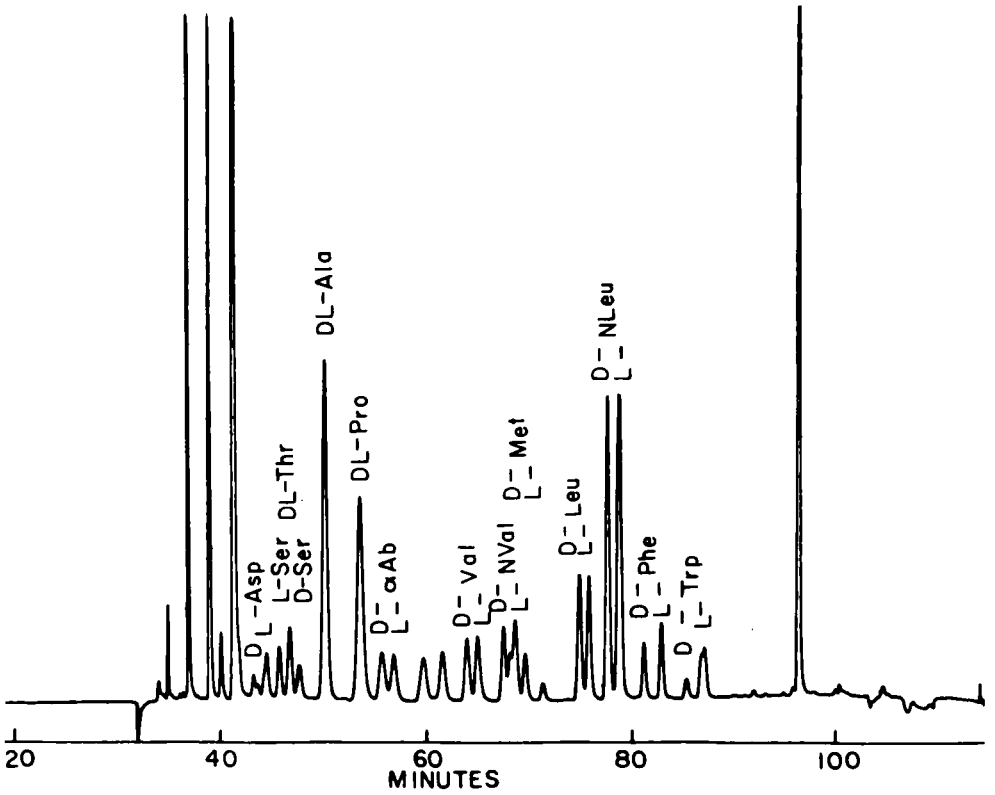

Figure 1. Separation of D,L-amino acid standards. Mobile phase: 5.0 mM L-histidine methyl ester, 2.5 mM $CuSO_4.5H_2O$ and 2.0 g of ammonium acetate, pH 5.5. A stepwise gradient was formed by blending the buffer with a 40% acetonitrile solution of the same buffer.

amino acids. It has long been known that primary amino compounds react stoichiometrically with OPA in alkaline medium in the presence of a thiol. The thiol adds rapidly to OPA, forming an addition product that is subsequently attacked by the primary amine. The reaction is fast, goes to completion in less than two minutes, with virtually no detectable by-products: unlike acylating reagents such as dansyl chloride, OPA reacts with the amino group, not the hydroxyl group of

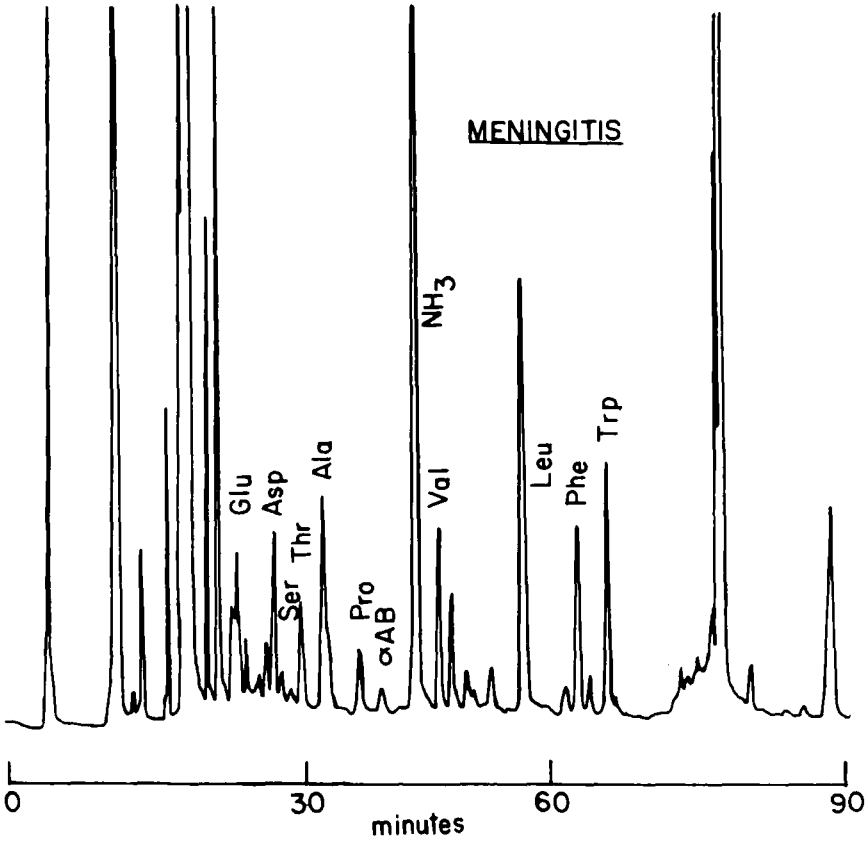

Figure 2. Amino acid profile of CSF from a patient with meningitis.

tyrosine. The product is highly fluorescent while the reagents are not, thus offering excellent detection sensitivity. Post column derivatization with OPA has become a favorite method for detecting amino acids emerging from HPLC ion exchange columns. A variety of thiols form adducts with OPA (20). For detection of amino acids after HPLC, the thiol generally used is mercaptoethanol. Precolumn derivatization with OPA and mercaptoethanol, followed by HPLC of the derivatives, has also

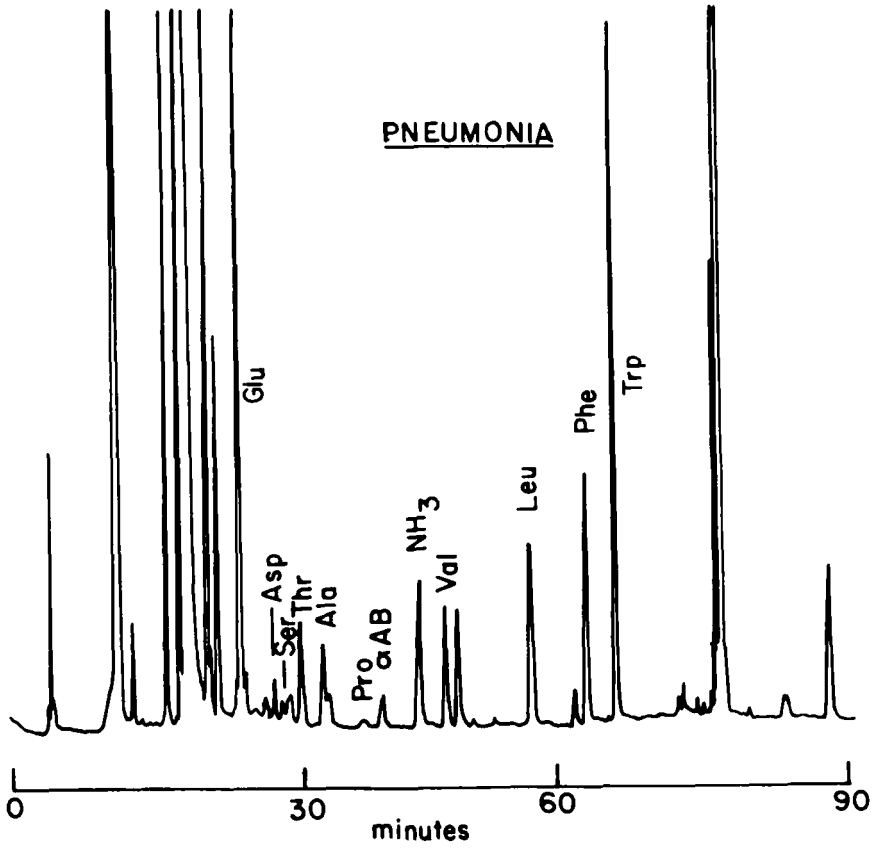

Figure 3. Amino acid profile of CSF from a patient with Pneumonia.

been used(21,22). Aswad (23) reported substituting N-acetyl-L-cysteine for mercaptoethanol, thus transforming the OPA derivatives from enantiomers to diastereoisomers, in the hope of resolving enantiomers. With conventional HPLC on a reversed phase column and a water-acetonitrile gradient, the only isomeric pair resolved was D,L aspartic acid.

Examination of molecular structure of the isoindole obtained by reacting OPA with N-acetyl-L-cysteine and an amino

TABLE 2

Capacity Ratio (k') and Selectivity (α) of Amino Acids Derivatized with O-phthalaldehyde in the Presence of N-Acetyl-L-Cysteine.

	Amino Acid	k'_L	k'_D	α
1	Asp	1.9	2.5	1.3
2	Glu	5.8	9.6	1.6
3	Ser	17.8	24.3	1.4
4	Asn	22.4	31.0	1.4
5	Thr	48.6	58.7	1.2
6	Lys	8.2	12.4	1.5
7	Cit	8.6	13.8	1.6
8	Ala	11.5	15.6	1.4
9	Arg	17.0	22.9	1.3
10	Tyr	6.2	9.8	1.6
11	Val	9.0	16.5	1.8
12	Met	16.3	19.7	1.2
13	Nval	17.2	22.2	1.3
14	Etho	37.2	45.7	1.2
15	Try	39.9	60.1	1.5
16	Phe	43.0	48.2	1.1
17	Leu	48.4	65.4	1.4
18	Nleu	48.6	62.1	1.3

Mobile phase: 2.5 mM Cu(II) L-proline with no acetonitrile for 1-5, 5% acetonitrile for 6-9, and 10% acetonitrile for 10-18.

acid suggested that the carboxylate and the nitrogen of the N-acetyl amino group of cysteine could form bi-dentate Cu(II) complexes. We therefore derivatized primary amino acids with OPA in the presence of N-acetyl-L-cysteine, injected them onto a reversed phase column using L-proline Cu(II) as the mobile phase and resolved the optical isomers (Table 2, Figure 4) (17).

By substituting mercaptolethanol for NAC, the chiral separation disappeared. This confirmed that the chiral separation we were achieving resulted from mixed chelate complexation at the carboxyl group and the N-acetyl amino group

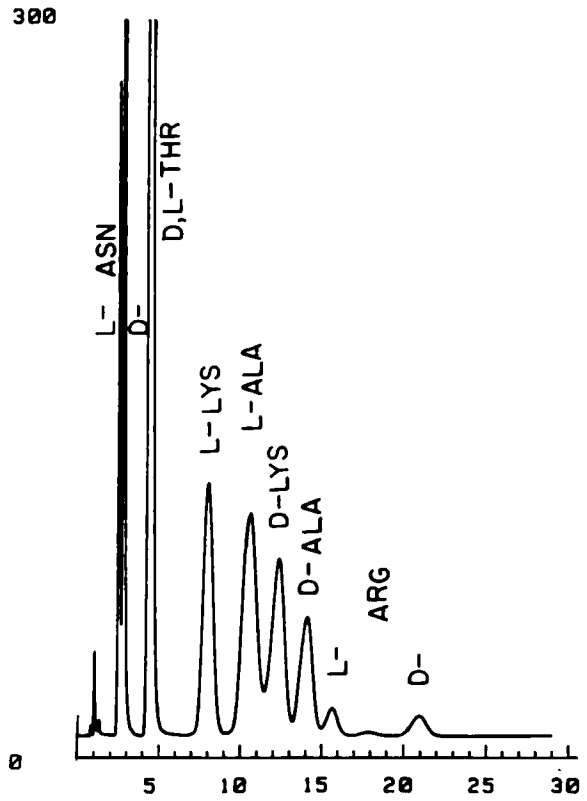

Figure 4. Chiral separation of polar amino acids as the OPA derivatives. Mobile phase: 5.0% acetonitrile in a buffer containing 5 mM L-proline, 2.5 mM $CuSO_4.5H_2O$ and 2.0 g ammonium acetate, pH 7.0. Flow rate 2.0 ml/min.

of the N-acetyl-L-cysteine on the derivative rather than that of the analyte amino acid. We therefore tried to separate enantiomers of the derivative of phenylethylamine, as an example of a primary amine with no other functional group, with OPA-NAC and achieved immediate success. We followed this with pilot separations with normetanephrine as an example of an amino alcohol, again with success (Figure 5).

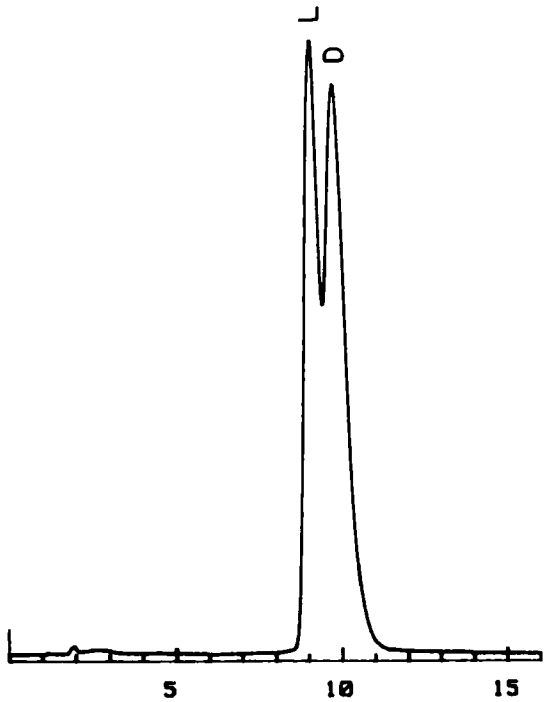

Figure 5. Chiral separation of D and L-Normetanephrine as the OPA derivatives. Mobile phase: 15% acetonitrile in a buffer containing 5 mM L-proline, 2.5 mM $CuSO_4.5H_2O$ and 2.0 g ammonium acetate, pH 7.0. Flow rate 2.0 ml/min.

Because of the dependence of the formation of the OPA adduct on the presence of a thiol group, we also studied the possibility of developing a procedure specific for enantiomers of sulfhydryl compounds. N-acetyl penicillamine was chosen as an example of a sulfhydryl compound, (N-acylation prevents reaction with the amine function). This was reacted with OPA in the presence of L-arginine as source of the chiral amine function and with no other source of sulfhydryl agent. Clear separation of the D and L penicillamine derivatives was achieved with reversed phase HPLC and mobile phase containing Cu(II) and L-proline (Figure 6).

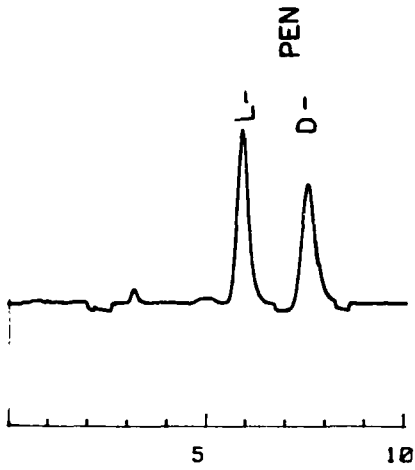

Figure 6. Chiral separation of N-acetyl-D,L-penicillamine as the OPA derivatives. Mobile phase: 10% acetonitrile in a buffer containing 5 mM L-proline, 2.5 mM $CuSO_4.5H_2O$ and 2.0 g ammonium acetate, pH 7.0. Flow rate 2.0 ml/min.

RESOLUTION OF D AND L IMINO ACIDS

The simultaneous detection and resolution of enantiomers of secondary amino acids is a more complicated problem. N-dansylating results in highly fluorescent derivatives but the optical isomers are not resolved by the complexation methods, possibly because the derivative, which contains a tertiary amino group, does not form the proper complex with copper(II) (4,12). Analysis of free amino acids using the copper system followed by post-column reaction with O-phthalaldehyde for detection, results in clear separation of enantiomers, but the secondary amino acids, which do not form fluorescent derivatives with O-phthalaldehyde (5,6) are difficult to detect. A procedure which uses low concentrations of Cu(II) L-aspartame (8,9) as the resolving agent, although not as sensitive as detection by fluorescence of the dansyl

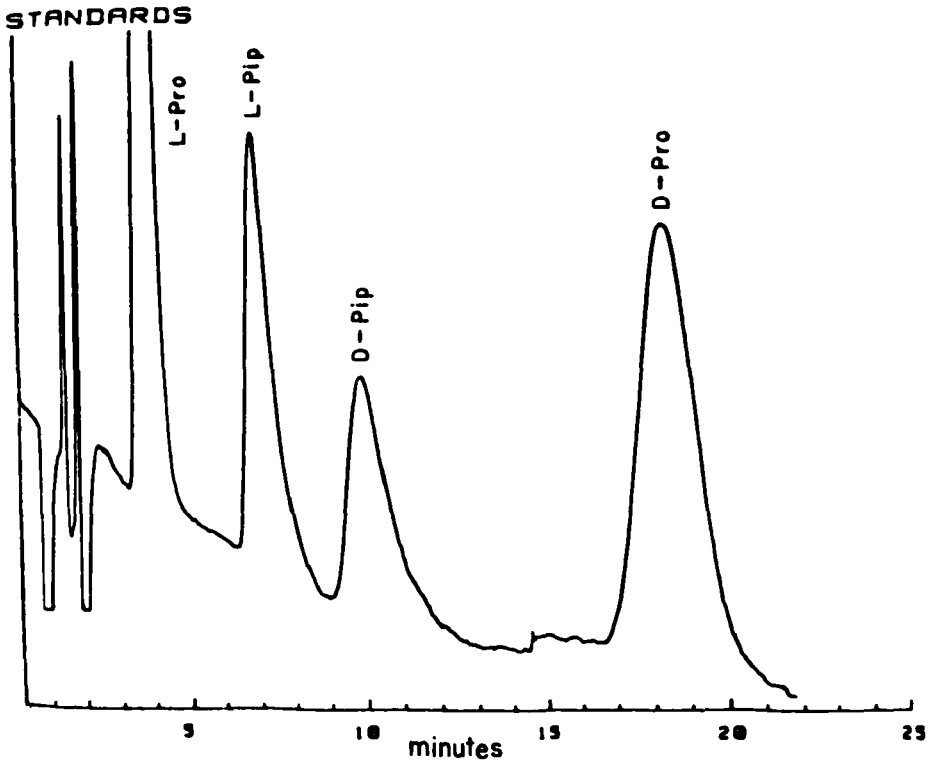

Figure 7. Separation of stereoisomers of proline and pipecolic acid. The Nucleosil 5 C18 column, 150 x 4.2 mm, was equilibrated with "loading buffer" containing 295 mg L-aspartame and 100 mg of copper sulfate in 1 liter of water. Mobile phase: 100 mg of copper sulfate and 25 ml of loading buffer diluted to 1 liter with water. Flow rate: 1.5 ml/min.

derivatives, is a useful compromise. Both D,L-proline and D,L-pipecolic acid are resolved and detected (Figure 7).

Patients with at least three genetic diseases: Zellweger's disease, hyperpipecolatemia and familial hyperlysinemia, excrete excessive quantities of pipecolic acid in their urine (24). Pipecolic acid found in the urine has been assumed to be the L isomer. We have analysed D,L-pipecolic in urine by

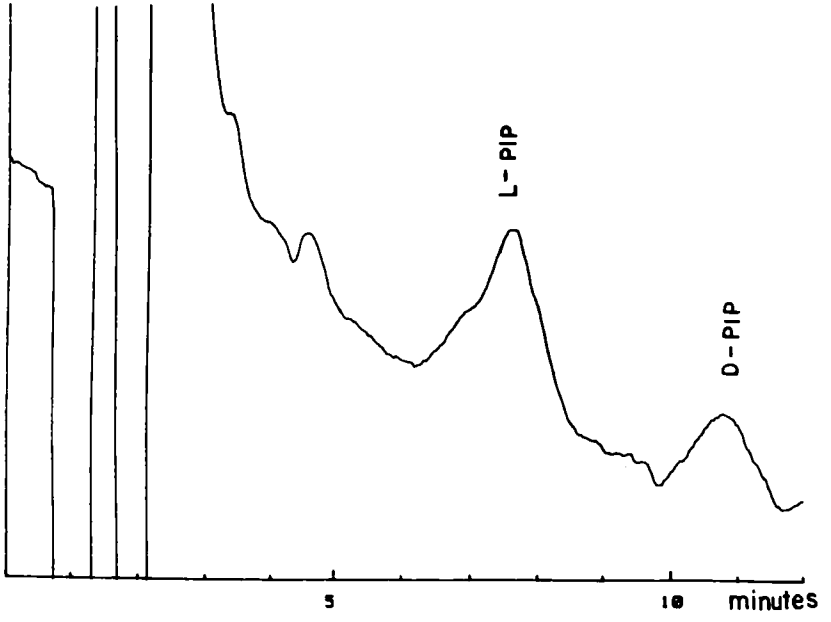

Figure 8. D and L pipecolic acids in urine of a patient with hyperlysinemia.

rechromatographing the pipecolic fraction from an ion exchange column on a reversed phase column by the Cu(II)-L-aspartame mobile phase as the mixed chelate complexes. Both D and L isomers were found present in the urine of normal patients who ingested a large quantity of L isomer, demonstrating racemization in vivo. Both isomers were also present in the urine of patients with hyperlysinemia with high levels of pipecolic acid while urines from patients with Zellweger's disease had high levels and only the L isomer (Figure 8). It is believed that this is the first observation that the D isomer of a natural amino acids is found in any excess amounts in humans.

STEREOSELECTIVITY

The stereoselectivity of the isomers of an amino acid depends on the stabilities of the ternary Cu(II) complexes. Resolution of the two diastereomers results because complexes of different stability have different chromatographic properties. The mixed chelate complex of one of the two isomers that has the higher stability will be retarded more on the column. A simple illustration of this is the L-proline copper complexes (Figure 9). In solution, the bis(amino) acids coordinate around the metal in a trans conformation with two water molecules in the axial position. The lesser stability of the L-L complex is apparently due to the interaction of the & substituent of the dansyl amino acid and the pyrrole ring of proline with the apically coordinated water molecule all in the same plane.

When using metal complexes of amino acids with chelatable side chains in the mobile phase, complex stability due to side chain interactions will also influence stereoselectivity because these side chains also participate in coordination with the metal ion and electrostatic interaction with the analyte ligand. L-arginine, for example is a chiral ligand with a side chain. L-arginine and the analyte amino acid coordinates around Cu(II) in the cis conformation allowing charge interaction of the positive guanido group of arginine with the

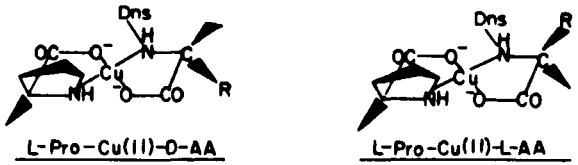

Figure 9. Ternary complexes of L-proline and D,L isomers of amino acid with Cu(II).

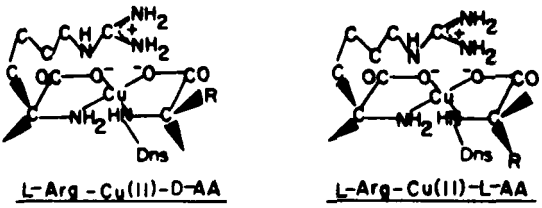

Figure 10. Ternary complexes of L-arginine and D,L isomers of amino acid with Cu(II).

negative carboxyl group of the neighboring dansyl amino acids. Electrostatic ligand-ligand interactions within the complex molecule is responsible for the more stable L-arginine-Cu(II)-L-amino acid ternary complex and retained the L isomer longer in the column (Figure 10).

CONCLUSION

Procedures are described for the resolution of the optical isomers of all common amino acids and imino acids. Most of these applications are simple to use. Stereoselective chromatography by mixed chelate complexation can be implemented on the reversed phase system by simply replacing the mobile phase with one that contains chiral Cu(II) complex.

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