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STEREOSELECTIVE D- AND L-AMINO ACID ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

This paper describes a gradient system for separating D- and L-isomers of Dns-amino acids by mixed chelate complexation through the addition of histidine methyl ester and copper sulfate to the mobile phase. Most of the biologically important amino acids were separated in a single analysis. With a simple solvent gradient consisting of increasing concentrations of acetonitrile in L-histidine methyl ester buffer all the common amino acids were resolved except cysteine and all optical isomers were resolved except those of threonine, alanine and proline. Analysis time was 90 min. Use of this system for determining non-protein amino acids in human cerebrospinal fluid showed the amino acids to be L-isomers, as expected. The pattern in fluid from a patient with bacterial meningitis was different from that of most of the others.

INTRODUCTION

In the now classical assay described by Stein and Moore[1]. amino acids were resolved using two ion-exchange columns. The currently standard procedures use ion-exchange chromatography followed by derivatization post-column with with ninhydrin[2–5] or *o*-phthalaldehyde (OPA)[6,7]. More recently many of the common amino acids have also been resolved by reversed-phase high-performance liquid chromatography (HPLC) of OPA or Dns (5-dimethylamino-naphthalene-1-sulfonyl)[8,9] derivatives. In these procedures D- and L-isomers of the various amino acids are not resolved.

A number of approaches for resolving optical isomers of amino acids have been proposed[10–12]. In most of these, resolution is generally based on the different behavior of the isomers in ligand exchange, crown ether complexation, charge transfer complexation, hydrogen bonding interaction or metal complex formation.

In this last technique, a non-polar, reversed-phase column is usually used with a mobile phase containing chiral metal complexes. LePage *et al.*[13] and Lindner *et al.*[14] used L-2-alkyl-4-octyldiethylenetriamine complexes of zinc and other metals in the mobile phase to separate Dns derivatives of the amino acids. Hare and Gil-Av[15] who previously reported using Cu(II)–proline eluents to separate free D- and L-amino acids, more recently studied Cu(II)–di-N-propylalanine complexes[16]. Grushka and

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co-workers[17–19] using complexes of aspartame and derivatives of aspartic acid, resolved a number of D- and L-amino acids.

We have previously described methods for separating D- and L-Dns-amino acids on reversed-phase columns[20,21]. An optically active copper chelate added to the mobile phase enables the two labile, mixed diastereoisomers in the racemic mixture to be resolved. The system separates the different amino acids from one another along with separating their optical isomers. Some chiral additives, such as proline and histidine, are more selective for optical isomers while others, such as histidine methyl ester offer better separations of the different amino acids. It is thus possible to select the appropriate reagent for achiral hydrophobicity or chiral selectivity for separating many common amino acids and their optical isomers in a single chromatographic run.

In this paper, we describe the use of a mobile phase containing histidine methyl ester–copper(II) complex for the achiral separation of Dns-amino acids and the chiral separation of the respective D- and L-enantiomers in a single run, achieving the separation that the current amino acid analyzers failed.

EXPERIMENTAL

Instrumentation

The chromatograph consisted of two Altex 110A pumps and a Model 420 gradient microprocessor (Altex Scientific, Berkeley, CA, U.S.A.). Samples were introduced through a Rheodyne 7105 injection valve. The analytical columns were 15 × 0.42 cm packed with Nucleosil C_{18} by the downward slurry technique. Detection of the Dns amino acids was by the Fluoro-tec filter fluorometer (American Research Products, Kensington, MD, U.S.A.). The amplified detector signal was read out on a Model 4416 data system (Nelson Analytical, Cupertino, CA, U.S.A.) and a Model 56 chart recorder (Perkin Elmer, Norwalk, CT, U.S.A.).

Reagents

Acetonitrile distilled in glass was bought from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), D- and L-Dns-amino acids were purchased from Sigma (St. Louis, MO, U.S.A.) and Pierce (Rockford, IL, U.S.A.). The Dns-amino acids were also prepared by the method described by Tapuhi *et al.*[22]. The amino acid standards (5 μg/ml) or patient samples (50 μl) were derivatized in a reaction vial by adding 25 μl of saturated lithium carbonate and 50 μl of Dns chloride in acetonitrile (250 μg/ml) and heating the vial at 60°C for 15 min. An aliquot was then injected into the chromatograph. The buffer was $5.0 \cdot 10^{-3}$ *M* of L-histidine methyl ester, $2.5 \cdot 10^{-3}$ *M* copper sulfate and 2.0 g of ammonium acetate (pH 5.5). The mobile phase was a stepwise gradient blended with the buffer and a 45% acetonitrile solution of the same buffer.

RESULTS

As in other reversed-phase analyses, the separation of the Dns-amino acids was also influenced by the concentration of acetonitrile in the mobile phase: with lower acetonitrile concentrations, solutes were retained longer. By increasing the con-

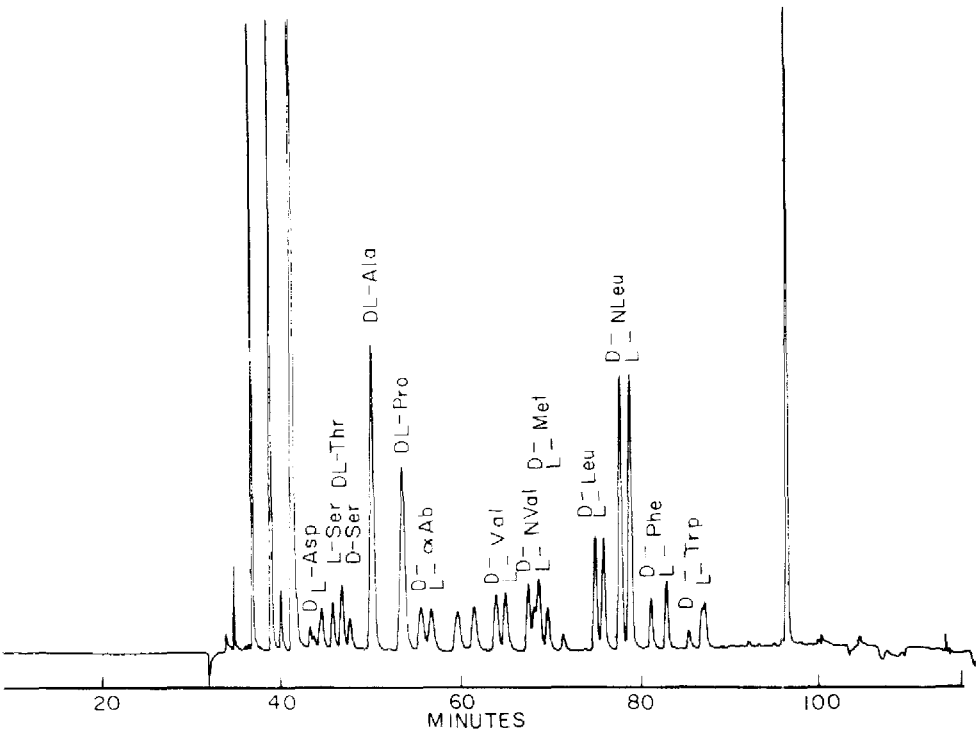

Fig. 1. Separation of D,L-amino acid standards. Mobile phase: $5.0 \cdot 10^{-3}$ *M* L-histidine methyl ester, $2.5 \cdot 10^{-3}$ *M* copper sulfate and 2.0 g of ammonium acetate (pH 5.5). A stepwise gradient was formed by blending the buffer with a 45% acetonitrile solution of the same buffer.

centration of acetonitrile in a prescribed gradient in buffer containing L-histidine methyl ester and Cu(II) in a 2:1 molar ratio, Dns-amino acids with aliphatic, aromatic and polar substituents were resolved (Fig. 1). The separation of the L-isomers alone is shown (Fig. 2). The identity of the peaks was confirmed by analyzing the individual amino acid isomer pairs. On the whole, good stereoselectivity was accomplished along with good separations of the different amino acids.

We applied this system to the determination of free, or non-protein, amino acids in human cerebrospinal fluid. Complete assays could be obtained starting with 50 μl of fluid, a volume which could generally be obtained from specimens received in our clinical laboratory for more routine analysis of glucose, protein and cell content. A patient with confirmed α-Streptococcus (Fig. 3) is compared to that of a negative culture (Fig. 4).

DISCUSSION

In the separation of amino acids by mixed chelate complexation we can assume the solutes and copper coordinate in 2:1 ratio in the mobile phase as follows:

$$2\,Ax + Cu(II) \rightleftharpoons Ax_2Cu(II) \quad (1)$$

$$2\,Ay + Cu(II) \rightleftharpoons Ay_2Cu(II) \quad (2)$$

$$Cu(II)Ax_2 + Cu(II)Ay_2 \rightleftharpoons 2\,Cu(II)AxAy \quad (3)$$

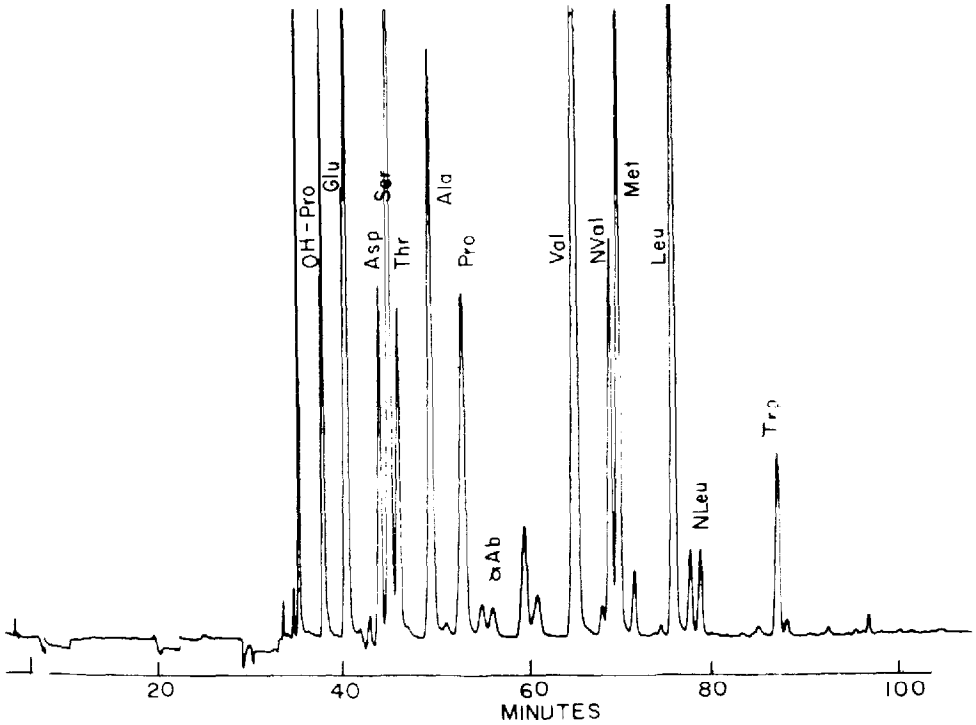

Fig. 2. Separation of L-amino acid standards, mobile phase as in Fig. 1.

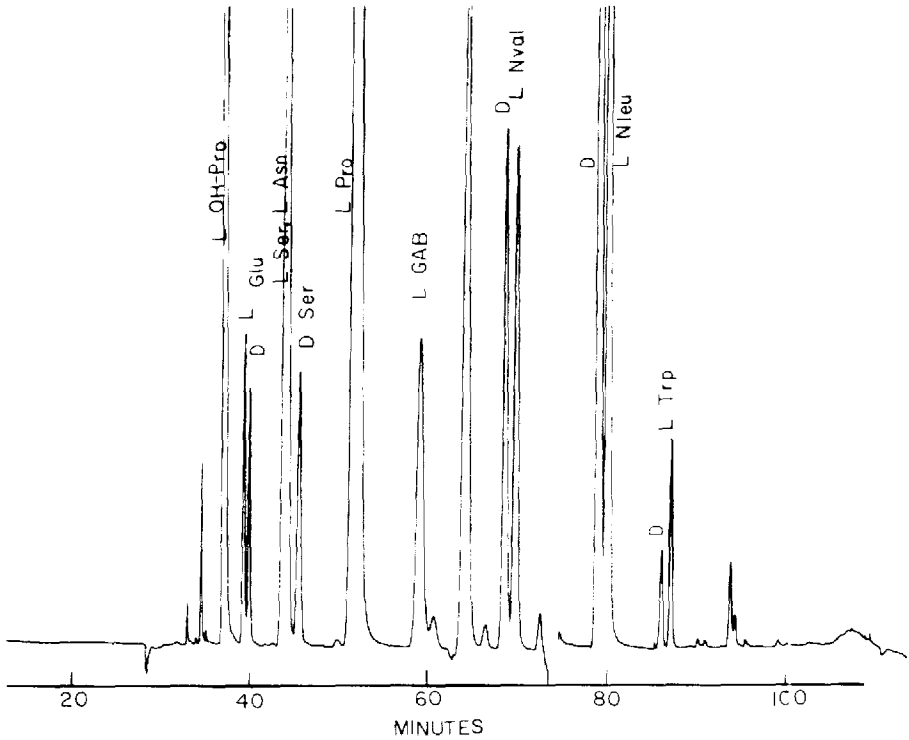

Fig. 3. Cerebrospinal fluid amino acid profile of a confirmed meningitis patient, mobile phase as in Fig

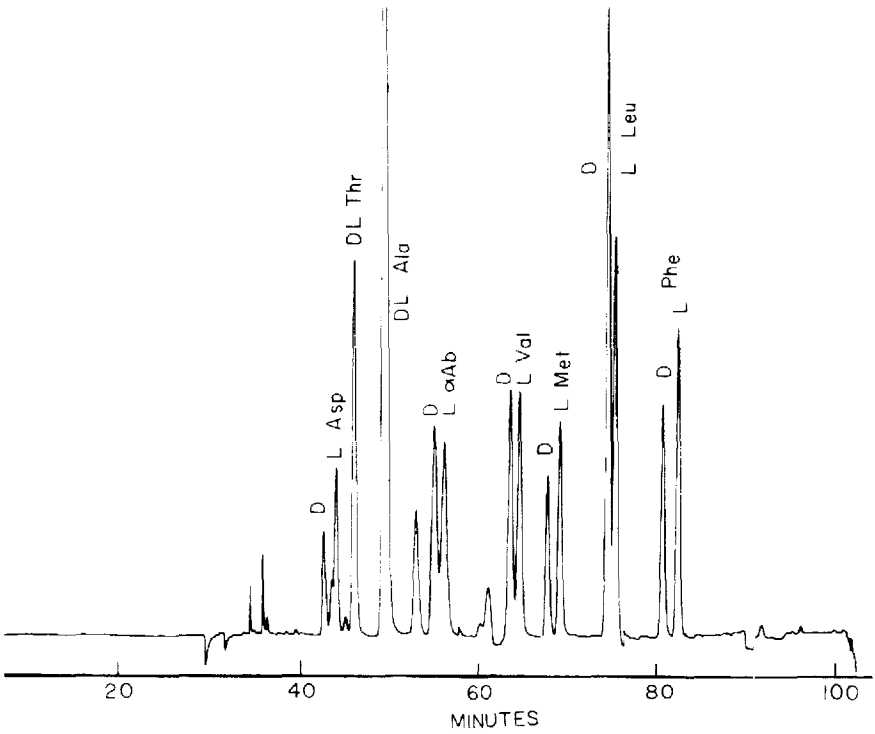

Fig. 4. Cerebrospinal fluid amino acid profile of a patient with negative culture, mobile phase as in Fig. 1.

Binary as well as ternary amino acid–copper complexes can be formed. At neutral pH, the reaction shown in eqn. 3 is preferred and as much as 70% of the copper species is in the form of ternary, mixed chelate complex. By adding an optically active chelate to the mobile phase, two mixed ligand diastereoisomers form which have different chromatographic properties and can be separated as mixed chelates in the chromatographic system.

The work described here is a direct outgrowth of the observation in previous studies that some copper(II) complexes are very selective towards D- and L-isomers, while others give better overall separation of amino acids and D- and L-isomers. Histidine methyl ester is an example of the latter. In this work, we optimized the last system by using a carefully controlled gradient of acetonitrile in buffer. Most of the amino acids were separated and their isomers resolved in the same analysis. As we reported previously, the DL-isomers of proline, alanine and threonine were not resolved. The separations between various L-isomers are superior in some instances to those by simple reversed-phase chromatography with an isocratic mobile phase. The gradient used here can be modified easily for specific applications by changing either the copper(II) complex or the acetonitrile concentration.

The resolution in mixed chelate complexation chromatography is pH dependent. Change in pH of the mobile phase shifts the equilibrium distribution of the various metal species in solution. At pH 7, the resolution of the D- and L-isomers is

close to unity. Using a gradient that has a lower starting pH, the resolution was less but amino acids pairs like proline–alanine and serine–threonine were resolved. This is the major advantage of the mobile phase containing histidine methyl ester: at pH 5 almost all of the mixed chelates we tried failed to give adequate resolution while the resolution of histidine methyl ester persisted.

The order of elution of the different amino acids can be summarized as follows: (1) the acidic amino acids; (2) the small and polar hydroxylated amino acids; (3) the long chain alkyl substituted and aryl amino acids; (4) the di-Dns-amino acids. The elution behavior and selectivity is caused by differences in the stability of the mixed complex on one hand an the interaction of the mixed complex with the hydrocarbon stationary phase on the other. Thus hydrophobic interaction causes longer retention of the larger amino acids and the straight chain isomer norvaline over the branched chain valine. The dependence of the retention behavior on the stability of the mixed complexes is demonstrated by the retardation of the L- over the D-enantiomer. Since the selectivity of this type of system depends on both hydrophobicity and complex stability, one can use solvent or metal complex concentration gradients to facilitate the separation. The gradient profile can be easily manipulated. If separation of a narrower group of amino acids is required, a gradient that offers much faster analysis can be chosen.

Tapuhi *et al.*[23] approached this problem by the use of a two column technique in which one column separates the individual amino acids, while the second column separates the optical isomers. The DL-pair to be separated is transferred from the first column into the second column by an on-line switch valve. Since two chromatographic systems are required, turn around time for any one pair of optical isomers is determined by the time of elution in the chiral system. In the system described here we accomplished the separation with a standard HPLC system with gradient capability and a fluorescence detector. As many as 14 optical isomers were resolved in a 90-min analysis while the switch column technique can resolve only 2 amino acids in the same time. Unlike ligand-exchange chromatography, the 15-cm reversed-phase column is readily available. The efficiency of the column, generally significantly better than can be achieved with ligand-exchange columns, is maintained when eluting with the mobile phases we described.

The system proved quite reproducible even though a gradient was employed. When four aliquots of the standard were derivatized and chromatographed separately, the retention times reproduced within less than 0.5% (R.S.D.). The peak areas reproduced in most instance to within 5% (R.S.D.) except where peaks overlapped.

The amino acid profile of a patient with meningitis caused by α-Streptococcus gave a distinctive amino acid profile. As expected, most of the amino acids of cerebrospinal fluid were in the L-form.

CONCLUSION

Gradient elution with a copper(II) complex of histidine methyl ester on a reversed-phase column appears to offer a viable means for amino acid analysis with DL-isomer separation on a single column. The separation is highly selective, giving excellent overall amino acid separation and optical resolution. The system is reproducible. It has all the advantages of reversed-phase chromatography with the extra dimension of separation via metal complexation.

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