Determination of Enantiomers of Amino Acids by Reversed Phase High Performance Liquid Chromatography

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The chromatographic resolution of enantiomeric amino acids is accomplished on reversed phase columns using aqueous mobile phases containing the chiral reagent L-aspartylcyclohexylamide–Cu(II) [AspcHex–Cu(II)]. This reagent has the additional advantage that the copper ions allow the detection of nonaromatic amino acids at 230 nm. The separation seems to be a result of hydrophobic interactions between the cyclohexylamide moiety of the chiral additive and the side chain of the amino acids. The results indicate that the composition of the chiral reagent in the mobile phase is $(AspcHex)_2Cu(II)$ and that the amino acids must replace one of the AspcHex for the resolution to occur.

Many attempts have been made in the past few years to resolve enantiomeric mixtures by liquid chromatography. Some of the studies are summarized in recent reviews by Krull (*1*) and by Davankov and Semechkin (*2*). Much of the effort has concentrated on the separation of enantiomeric amino acids, mainly because of their biological importance and the significance in determining the extent of racemization in protein and peptide synthesis. The advantages of liquid chromatographic techniques in such separations are many; for example, the ability to resolve, in one experimental run, a mixture of enantiomers; the minimal requirement of pretreatment steps before the separation; and the ability to use such techniques in conjunction with amino acid analyzers.

Enantiomers can be resolved by conversion into diastereomers. Indeed, diastereomers have been separated by both gas and liquid chromatography with little difficulty (*1*, *3*, *4*). A more elegant approach is a system where the diastereomers are formed in situ. In order to fulfill such a requirement, an optically active reagent must be present as part of either the mobile phase or the stationary phase. This approach was first applied successfully in gas chromatography by Gil-Av and co-workers (*5*).

Concurrently with the gas chromatographic separations, several groups have begun investigating the utility of liquid chromatography (LC) for resolving amino acid enantiomers. Among the early works are those of Angelici and his group (*6*) and of Davankov (*2*, *7*, and references therein), who used amino acid derivatives, for example, proline, grafted to polymeric beads as the chiral stationary phase. To achieve separation, transition metal ions, such as Cu(II), were added to the mobile phase to be absorbed by the covalently-bound amino acids. The resolving power of such systems is attributed to the stereoselectivity of the complex formation between the D- or L-amino acid with the Cu(II)–proline moiety. More recently, Lefebvre et al. (*8*), Tsuchida et al. (*9*), and Gubitz et al. (*10*) have resolved enantiomers in a similar fashion. Baczuk et al. (*11*), Hara and Dobashi (*12*), Pirkle and House (*13*), and Sousa et al. (*14*) have resolved enantiomers of amino acids, or their derivatives, without the use of metal ions.

The studies described above used the chiral center, which forms the diastereomers with the amino acids, as part of the stationary phase. An alternate approach, however, involves the addition of the resolving agent to the mobile phase. There are very few literature reports using this approach. Yoneda and Yoshizawa (*15*) have used sodium tartrate-*d* to separate optically active tris complexes of amino acids with Co(III). Quite recently Karger and his co-workers (*16*), Hare and Gil-Av (*17*), and this group (*18*) have all described the use of chiral eluents for the resolution of amino acid enantiomers.

Our previous studies on the use of metal ions in either the stationary (*19*) or mobile phase (*20*) led us to the conclusion that metal complexes of chiral reagents in the mobile phase should provide the selectivities needed for enantiomeric resolution.

In our initial work, the chiral reagent was either the Cu(II) or Zn(II) complex of L-aspartyl-L-phenylalanine methyl ester (Aspartame). The reasons for using this reagent are given elsewhere (*18*). However, it is important to repeat here that the complexes it forms with metal cations consist, most likely, of a six-membered ring which involves the β-carboxy and α-amino groups of the aspartyl. Such a six-membered ring is less stable than the usual five-membered ring obtained when an α-carboxy and α-amino groups bond to the metal ions. As a consequence, the amino acids to be separated can form a stronger complex with the metal ions and displace the Aspartame, a fact that is crucial for the resolution.

Our study has shown (*18*) that Aspartame-Cu(II) or Aspartame-Zn(II) could be used to resolve racemic mixtures of aromatic amino acids. It is desirable (a) to extend the method to other amino acids, (b) to investigate the role of the phenylalanine and methyl ester residues in the separation, and (c) to ascertain the necessity of two asymmetric centers in the resolving agent.

EXPERIMENTAL

Apparatus. A Spectra-Physics model 8000 liquid chromatograph was used in this study. The UV detector was operated mostly at 230 nm. The mobile phase flow rate was 2 mL/min at a pressure of about 2500 psi. The column was 25 cm long by 4.1-mm i.d. The stationary phase was ODS bonded to Partisil 10. It was prepared by refluxing chlorodimethyloctadecyl silane with the silica gel in dry toluene.

The mobile phase was an aqueous mixture of Aspartame–Cu(II) complex or L-aspartylcyclohexylamide–Cu(II) complex.

Preparation of L-Aspartylcyclohexylamide (AspcHex). ZAsp(OBzl)OH (3.7 g, 10.2 mmol) was dissolved in dimethyl formamide (DMF; 25 mL). *N*-Methymorpholine (1.13 mL) was added and the mixture was cooled to −15 °C. Isobutyl chloroformate (1.26 mL, 9 mmol) was added and after 2 min, freshly distilled cyclohexylamine (1.15 mL, 10 mmol) was also added to the reaction. The mixture was kept 1 h at −15 °C and then at 0 °C. $KHCO_3$ (2 M, 15 mL) was added under vigorous stirring. After 0.5 h, a solution of NaCl (25% 100 mL) was added. The white precipitate was collected by filtration and washed with water

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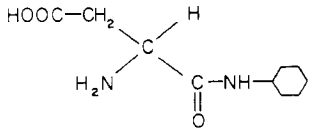
Table I. Capacity Ratios and Selectivities of Some Amino Acid Enantiomers at Three Concentrations of AspcHex-Cu(II) in the Mobile Phase

	1×10^{-3} M			2.5×10^{-4} M			1×10^{-4} M		
	k' (L)	k' (D)	α	k' (L)	k' (D)	α	k' (L)	k' (D)	α
Pro	0.24	1.12	4.46	0.55	1.42	2.58	2.17	3.0	1.38
Val	0.41	0.82	2.0	1.07	1.52	1.42	1.83	2.17	1.18
Nvl	0.56	0.89	1.59	1.14	1.43	1.25	1.74	2.0	1.15
Cys	0.76	1.15	1.51	1.81	2.43	1.34	--	--	--
Met	1.41	2.06	1.46	2.52	3.29	1.30	3.7	4.4	1.19
Dopa	1.78	2.33	1.31	3.21	3.90	1.22	5.67	6.86	1.21
Ilu	1.76	3.76	2.14	2.86	5.0	1.75	4.5	7.0	1.55
Leu	2.35	3.88	1.65	3.23	4.63	1.43	5.19	6.81	1.31
Nlu	2.47	4.23	1.71	3.67	5.40	1.47	5.13	6.76	1.32
Tyr	3.03	4.5	1.48	5.02	6.98	1.39	7.03	8.86	1.26
Eth	4.65	6.64	1.43	7.14	9.43	1.32	10.7	12.8	1.2

until pH 7 was reached, and no traces of Cl^- were found in the wash. The product was then dried. The yield was 85% and its melting point was 139–141 °C. Elemental analysis performed on the product showed C, 68.23%; H, 7.02%; N, 6.71%. The calculated values for the product ($C_{25}H_{32}N_2O_5$) are C, 68.47%; H, 6.90%; N, 6.39%. To a solution of ZAsp(OBzl)-cHex (3 g) in acetic acid (90% 30 mL), Pd/C 10% (300 mg) was added and the mixture was hydrogenated at room temperature (5 atm). The catalyst was removed by filtration and the solvent was evaporated. The residue was recrystallized from water with a yield of 86%, and exhibited a melting point of 235 °C. Elemental analysis of the product found C, 56.12%; H, 8.32%; N, 13.23%. The calculated values for $C_{10}H_{18}N_2O_3$ are C, 56.06%; H, 8.47%; N, 13.07%. The structure of AspcHex (I) is shown below.



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Chromatographic Studies. The mobile phases were prepared by dissolving the chiral reagent and Cu(II) to yield 10^{-4}–10^{-3} M solutions. When measuring separation factors, each of the enantiomers was injected individually.

Chemicals. All the amino acids as well as the Aspartame, were purchased from Sigma Chemicals. The water in the mobile phase was triple distilled in our laboratory. Spectroscopic grade acetonitrile was obtained from Aldrich.

RESULTS AND DISCUSSION

Studies with L-Aspartylcyclohexylamide (AspcHex). The importance of the presence of two chiral centers as well as the phenyl and methoxycarbonyl groups in the resolving agent Aspartame (*18*) must be studied in order to decipher the separation mechanism. For that purpose AspcHex was prepared. AspcHex has only one chiral center an it lacks phenyl and methoxycarbonyl groups. Instead the hydrophobic part consists of a cyclohexyl ring. AspcHex has two major advantages: (a) Owing to the absence of a phenyl group, the background of the detector signal should be smaller than with Aspartame. (b) Because of the low background absorbance, nonaromatic amino acids can be detected by virtue of the fact that they form with Cu a five-membered ring complex which strongly absorbs UV radiation at around 230 nm (*21*). This last point is of cardinal importance: the presence of AspcHex–Cu(II) complex in the mobile phase is found to allow not only the separation of the amino acid enantiomers but also their detection. Figures 1 and 2 demonstrate the effectiveness of AspcHex-Cu(II) in attaining racemic resolution and solute detection. In Figure 1 a mixture of D,L-methionine and D,L-ethionine were injected into the chromatograph and resolved with AspcHexCu(II). Figure 2 shows the enantiomeric separation of D,L-valine and D,L-leucine. In both chromatograms, the detection was done at 230 nm, and the amino acids

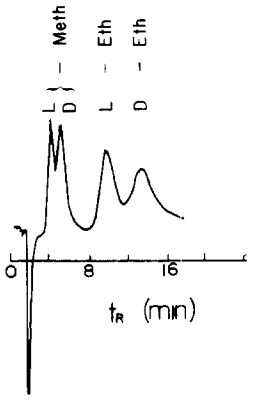

Figure 1. Enantiomeric separation of D,L-methionine and D,L-ethionine. The aqueous mobile phase contains 10^{-3} M AspcHex and Cu(II). T = 34 °C, flow rate = 2 mL/min. Detection: UV at 230 nm

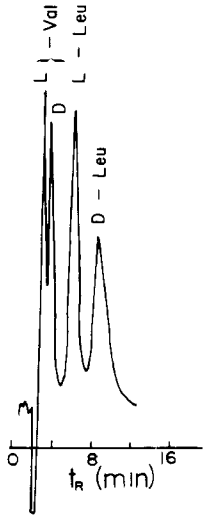

Figure 2. Enantiomeric separation of D,L-valine and D,L-leucine. Same conditions as in Figure 1

were injected as received from the vendor. This simplifies the anaylsis, since it does not require the post-column derivatization as was done by Hare and Gil-Av (*17*), or precolumn derivatization as was the case in the work of LePage et al. (*16*).

While the efficiency of the system was within the acceptable range, ($N > 4000/25$ cm), when tested with a standard mixture of aromatic hydrocarbons, it is low when measured from the chromatograms in Figures 1 and 2. The reasons for this, it is felt, are slow mass transfer processes. The efficiencies can be greatly improved by the use of a buffered mobile phase at the appropriate pH. However, no attempts were made to optimize the chromatographic resolution.

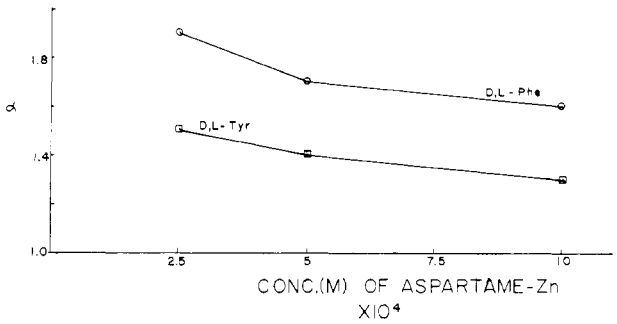

Figure 3. Effect of the concentration of Aspartame-Zn on the enantiomeric separations

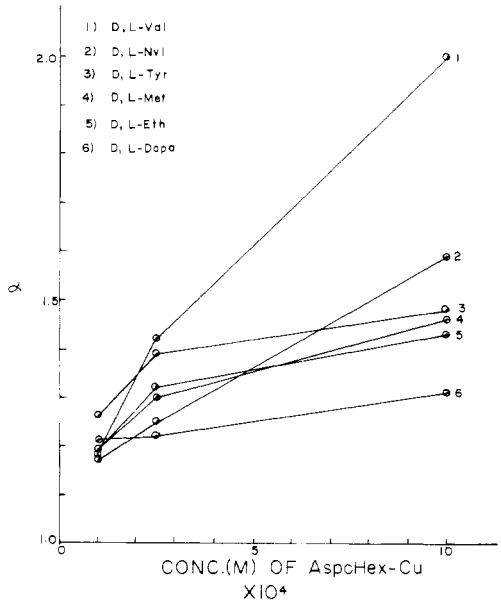

Figure 4. Effect of the concentration of AspcHex-Cu(II) on the enantiomeric separations

Table I demonstrates the ability of AspcHex-Cu(II) to resolve racemates. The separation factors of the enantiomers are at worst 1.3, and can be as high as 4.6. Such large α values indicate easy separations with modern liquid chromatographic equipment. Similar to the case of Aspartame, the L enantiomer elutes before the D one. Gil-Av found the same retention order with proline-Cu(II) resolving agent (*17*, *22*). Acetonitrile, 5%, was added to the mobile phase in order to elute the tryptophans. Without the organic modifier, these solutes were retained on the column for over an hour.

Effect of the Concentration of the Chiral Reagent. Our initial results indicated that the amount of the chiral reagent in the mobile phase affected the α values. Figure 3 shows the variation of α with the amount of Aspartame–Zn(II). Surprisingly the separation factors increased as the concentration of the reagent decreased over the range studied. The reason for this behavior of α is not clear although it is possible that at the concentration range shown in Figure 3, the stationary phase is saturated with Aspartame–Zn(II), and any decrease in the amount of these reagents shifts the partition equilibrium to the stationary phase.

The dependence of α on the concentration of AspcHex-Cu(II) is different from that of Aspartame–Zn(II) as shown in Figure 4. In some cases the decrease in the chiral reagent causes a drastic decrease in the selectivity. Table I shows that the capacity ratios increase as the concentration of AspcHex-Cu(II) is decreased. The retention order of either the L or the D isomers is also a function of that concentrate. The data tend to indicate that the amount of AspcHex-Cu(II) in the mobile phase is much greater than in the stationary phase.

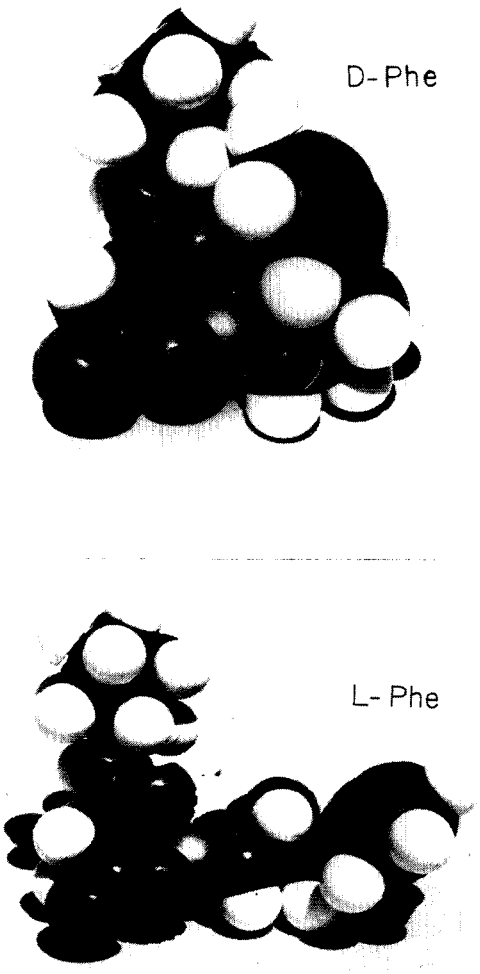

Figure 5. Space filling models of (AspcHex)-Cu(II)(L-phenylalanine) and (AspcHex)-Cu(II)(D-phenylalanine)

This point will have to be examined more carefully.

Interaction between Enantiomers and AspcHex-Cu-(II). It is clear from the results presented so far that a single asymmetric center in the chiral eluent is sufficient for the separation, and that the aromatic side chain as well as the methoxycarbonyl group can be replaced by a saturated ring. The question remains, however, as to the reason for the stereo recognition. It was pointed out by, among others, Feibush and Gil-Av (*23*) and Beitler and Feibush (*24*), that three contact points between the amino acids to be separated and the chiral reagent must be formed before the resolution can take place. Pirkle has recently amplified this "three contacts" theory (*25*). He has pointed out that the requirement for the contact points can be fulfilled by such interactions as charge transfer. The resolution described here can also be explained with the aid of the three contact points model. Two "points" are between the α-carboxy and α-amino groups of the amino acids and the Cu(II) in the AspcHex complex. The third association "point" is due to hydrophobic interactions between the side groups of the amino acids and those of the chiral resolving agent (be it AspcHex or Aspartame). Figure 5 shows space filling models of the ternary complexes (AspcHex)Cu-(II)–D-phenylalanine and (AspcHex)Cu(II)–L-phenylalanine. Only in the case of D-phenylalanine can the side chain of the amino acid interact with the cyclohexyl moiety of AspcHex. In an aqueous medium, the favored conformation is that which leads to the strongest interaction between the hydrophobic moieties of the ternary complex. The data in Table I seem

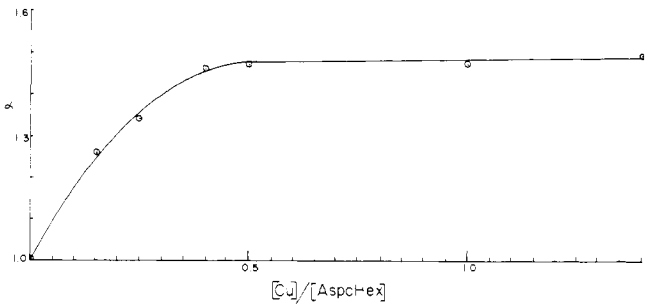

Figure 6. The α value of D,L-tyrosine as a function of the concentration ratio of Cu(II) to AspcHex

to support the above discussion. Alanine, with the smallest side chain, cannot be resolved under the condition described in Table I. Amino acids with bulky side chains such as valine and isoleucine show large α values. Amino acids with side chains capable of forming hydrogen bonding with the aqueous mobile phase, e.g., DOPA, in general, have smaller α values than the hydrophobic amino acids. One can speculate that the D-form of the amino acids elutes last, since the complex with the AspcHex-Cu(II) presents a more hydrophobic surface than that of the L isomer.

Effect of the Ratio of AspcHex to Cu(II). Workers in ligand exchange chromatography assumed a concentration ratio of two between the proline which was grafted on polymeric supports, and Cu(II). Hare and Gil-Av (*17*) have used the same concentration ratio of metal to proline in the mobile phase of their system.

There is ample evidence in the literature that Cu(II) bonds two amino acids via the conventional five-membered ring discussed before. It is possible that each Cu(II) ion complexes two AspcHex molecules, although six-membered rings are most likely involved in this case. The amino acids to be separated must replace one of the AspcHex units. This hypothesis is verified as follows. If the AspcHex-Cu(II) complex is necessary for the resolution, then changes in the [Cu]/[AspcHex] ratio should affect the α values. Without Cu(II), no resolution occurs. As Cu(II) is added and the AspcHex-Cu(II) is formed, the resolution increases. Now, if the composition of the resolving complex is $(AspcHex)_2Cu(II)$, then the α value should reach its maximum value at a [Cu]/[AspcHex] ratio of 0.5, and remain constant as the amount of copper is further increased, since additional Cu(II) is not part of the complex. On the other hand, if the main species in solution is AspcHex-Cu(II), then α would increase until the concentration ratio is unity. Figure 6 shows a plot of α values of D- and L-tyrosine vs. [Cu(II)]/[AspcHex]. The concentration of AspcHex was kept constant at 2×10^{-4} M, while that of the Cu(II) varied. It is clear that α increases until a ratio of 0.5 is obtained. Figure 6 seems to indicate that the complex in the mobile phase is of the general formula $(AspcHex)_2Cu(II)$; maximum resolution of enantiomers occurs when, in the mobile phase, a single Cu(II) is present for two molecules of AspcHex. Figure 6 is the chromatographic equivalent to the spectroscopic mole-ratio method of elucidating the composition of the complex ions in solutions. To our knowledge this is the first report of such a chromatographic approach to complex ion composition determination.

Implicit in Figure 6 is that the amino acids, which are injected into the column, replace one of the AspcHex molecules from the complex. For resolution to occur the diastereomers (L-AspcHex)Cu(II)(L-amino acid) and L-AspcHex)-Cu(II)(D-amino acid) must be formed.

Separation of Alanine and Histidine. Under the conditions of Table I, alanine and histidine could not be separated. However, when a phosphate buffer having a pH of 6 is used instead of distilled water, the enantiomers of both these amino acids can be resolved. For example, using this buffer with 3×10^{-4} M Cu(II) and 6×10^{-4} M AspcHex, the capacity ratios of L- and D-alanine are 2.14 and 2.63, respectively. This corresponds to an α value of 1.22. The capacity ratios of L- and D-histidine are 11.4 and 9.43, respectively, corresponding to an α value of 0.83. The elution order of the histidine enantiomers is to be noted; the D isomer is retained less than the L one. This might be explained by the fact that histidine forms a tridentate complex with Cu(II) via the imidazole group. Similar elution orders were found by Hare and Gil-Av (*17*).

AspcHex-Cu(II) seems to be an excellent chiral reagent for the separation of amino acid enantiomers. This complex not only provides the resolution, but it allows the detection of nonaromatic amino acids. The present results seem to indicate that stereospecific hydrophobic interactions between AspcHex-Cu(II) and the amino acids are necessary for resolution. Experiments are now under way in order to verify this theory. Attention should also be given to the relationship between relative peak areas of the enantiomers and sample size, temperature and amount of chiral reagent in the mobile phase.

Finally, we are now looking at the addition of Cu(II) ions for detection purposes in the routine analysis and separation of amino acids. In particular, we are studying the detection limit and its dependence on the concentration of the chiral reagent in the mobile phase.

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