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DETERMINATION OF THE ENANTIOMERS OF α-AMINO ACIDS AND α-AMINO ACID AMIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A CHIRAL MOBILE PHASE

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SUMMARY

A high-performance liquid chromatographic method for the enantiomeric analysis of a mixture of an α-amino acid and the corresponding acid amide is described. Reversed-phase chromatography with copper(II) acetate and N,N-di-*n*-propyl-L-alanine in the mobile phase are used for the separation. For Val and Val-NH_2, several parameters affecting retention and enantioselectivity were investigated. The results indicate that by manipulation of pH, ionic strength, temperature, concentration of Cu^{II}, N,N-di-*n*-propyl-L-alanine and ion-pairing reagent, good control of enantiomeric separation can be achieved. For α-amino acid amides a mechanism is proposed which may account for the retention and enantioselectivity. Examples of enantiomeric analysis of mixtures of α-amino acids and α-amino acid amides with aliphatic, aromatic and polar side-chains are given. The method can be used for the control of the enantiomeric purity of α-amino acids and the corresponding acid amides obtained by enantioselective synthesis.

INTRODUCTION

One of the routes to optically pure α-amino acids (α-AA) is through organic synthesis of racemic α-amino acid amides (α-AA-NH_2), followed by the use of a broad-specificity peptidase to achieve resolution on a large scale. In conjunction with this synthesis, analytical methods are required for the control of the enantiomeric purity of both α-AA and α-AA-NH_2. High-performance liquid chromatographic (HPLC) methods for the enantiomeric resolution of α-AA using a chiral copper complex as an additive to the mobile phase have been known for several years. Lindner and Hirschböck[1] recently summarized enantioselective HPLC systems generated by the mobile-phase additive mode. Copper complexes of N,N-di-*n*-propyl-L-alanine (L-DPA) were introduced by Weinstein *et al.*[2] and appeared to resolve the common protein amino acids. Davankov *et al.*[3] reported the resolution of valinamide into its enantiomers by ligand-exchange chromatography.

The aim of this work was to develop an HPLC method for the enantiomeric resolution of an α-AA together with the corresponding α-AA-NH_2. For this, we made use of copper complexes of L-DPA. We studied the influence of several mobile phase

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parameters in order to optimize the enantiomeric separation of the α-AA and the corresponding α-AA-NH_2. A model is proposed for the enantiomeric resolution of α-AA-NH_2 by the use of copper complexes of L-DPA.

EXPERIMENTAL

Materials

α-AA and α-AA-NH_2 were obtained from Sigma (St. Louis, MO, U.S.A.) or synthesized in our laboratory[4]. For each compound, both the racemic form and at least one optically pure enantiomer were available. L-DPA was prepared according to the method of Bowman and Stroud[5]. The pairing ion laurylsulphonate and *o*-phthalaldehyde (OPA) were obtained from Merck (Darmstadt, F.R.G.). 2-Mercaptoethanol (MCE) was supplied by Fluka (Buchs, Switzerland). Water was purified with a Milli-Q system. HPLC-grade acetonitrile and ethanol were obtained from Merck and used as supplied. All other chemicals were of analytical-reagent grade.

Instrumentation

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1081B liquid chromatograph, a Rheodyne (Cotati, CA, U.S.A.) 7010 injection valve for manual injection or a Micromeritics (Norcross, GA, U.S.A.) Model 725 autosampler for automated injection. The injection loop had a 20-μl capacity. The columns used were Nucleosil 120-C_{18} (250 × 4.0 mm I.D., 5 μm, and 125 × 4.0 mm I.D., 3 μm) and Polygosil 60-C_{18} (125 × 4.0 mm I.D., 5 μm) from Macherey, Nagel & Co. (Düren, F.R.G.) and Hypersil-ODS (200 × 4.6 mm I.D., 5 μm) from Hewlett-Packard. The flow-rate was 1 ml/min and the column temperature was kept at 40°C.

For reaction detection with OPA–MCE, the reagent was added to the column effluent by a mixing T. Post-column addition of reagent was effected with a Gilson (Villiers-le-Bel, France) Model 302 pump at a flow-rate of 1 ml/min. The OPA–MCE reaction was carried out in a coiled capillary stainless-steel tube (12 m × 0.35 mm I.D., coil diameter 12 mm) at 40°C. The fluorophores were monitored with a Waters Assoc. (Milford, MA, U.S.A.) Model 420 fluorescence detector. For excitation a 338-nm band-pass filter and for emission a 415-nm long-pass filter were used. Quantitation was performed with a Hewlett-Packard 3350 Laboratory Automation System.

Eluent, reagent and sample preparation

The mobile phase consisted of copper(II) acetate, L-DPA and triethylamine (TEA), titrated to the required pH with acetic acid. The concentration of the mobile phase components and the eluent pH varied, depending on the experiment, and are indicated in the figures. The OPA–MCE reagent was a mixture of potassium borate buffer (0.4 *M*, pH 10.0), OPA (6 m*M*), MCE (0.1%), ethanol (1.0%) and EDTA (30 m*M*). The chelating agent, EDTA, was added to the reagent in order to avoid precipitation of copper(II) hydroxide in the reaction coil under the alkaline conditions applied. The Cu(L-DPA)$_2$ solution consisted of copper(II) acetate and L-DPA in a concentration ratio of 1:2. Standards and samples were dissolved in the eluent.

RESULTS AND DISCUSSION

Effects controlling retention and enantioselectivity

With Val–Val-NH_2 and α-Ph-Gly–α-Ph-Gly-NH_2 as model compounds, we initially used the chromatographic conditions described by Weinstein *et al.*[2] to obtain enantiomeric resolution. Under these conditions, the α-AA-NH_2 showed tailing peaks and the enantiomers were only partially resolved. For the α-AA, good enantioselectivity was obtained ($\alpha > 2$). However, with Val–Val-NH_2, the L-Val-NH_2 peak overlapped with the D-Val peak.

In order to optimize the enantiomeric separation of a mixture of an α-AA and the corresponding α-AA-NH_2, we studied the influence of the following factors on the chromatographic behaviour of Val and Val-NH_2 enantiomers: TEA concentration, pH of the eluent, concentrations of Cu^{II}, L-DPA and Cu(L-DPA)$_2$, column temperature, ionic strength and concentration of laurylsulphonate in the eluent.

Effect of triethylamine

The peak tailing that we observed for α-AA-NH_2 may result from interactions of the polar amide function with free silanol sites on the reversed-phase column. A well known technique to suppress this tailing phenomenon is the addition of a competitive amine (*e.g.*, TEA) to the mobile phase. Fig. 1A shows the asymmetry factor (A_s) and resolution (R_s) of the enantiomers of Val-NH_2 as a function of TEA concentration from 0 to 5 m*M*. As shown by A_s, the peak symmetry of the enantiomers is improved by the addition of TEA to the eluent, and consequently the enantiomeric resolution increases. With respect to the capacity factors (k') and separation factors (α) of the enantiomers of Val and Val-NH_2, a decrease in these parameters can be seen when TEA is used in the concentration range 0.5–5 m*M* (Fig. 1B). Because TEA forms complexes with Cu^{II}, the TEA molecules will compete as ligands with the Val and Val-NH_2 enantiomers for the formation of the mixed-ligand complex, thus lowering k' with increasing TEA concentration. Use of a 5 m*M* concentration of TEA resulted in increased background noise and an unstable baseline. In subsequent experiments, we therefore used the minimum TEA concentration (0.5–1.0 m*M*) at which baseline resolution for the enantiomers of Val-NH_2 is obtained.

Effect of pH

For the enantiomeric separation of amino acids, Weinstein[6] suggested that ligand exchange may take place between the binary Cu(L-DPA)$_2$ complex and the enantiomers of the amino acids. In order to be coordinated as a bidentate ligand to Cu^{II}, the amino acid must possess an unprotonated amine group and an acid function in the anionic form[7–9]. With an α-AA-NH_2, coordination may take place via interactions of Cu^{II} with the unprotonated α-amino group and nitrogen of the acid amide group.

As a result, the formation of copper amino acid (amide) complexes will be strongly pH dependent. The influence of the eluent pH on the k' and α values of the enantiomers of Val and Val-NH_2 was studied over the pH range 3.5–5.5 (Fig. 1C). In order to avoid precipitation of copper(II) hydroxide, pH values above 5.5 were not studied.

The k' values of the Val enantiomers increase as the eluent pH rises. A similar

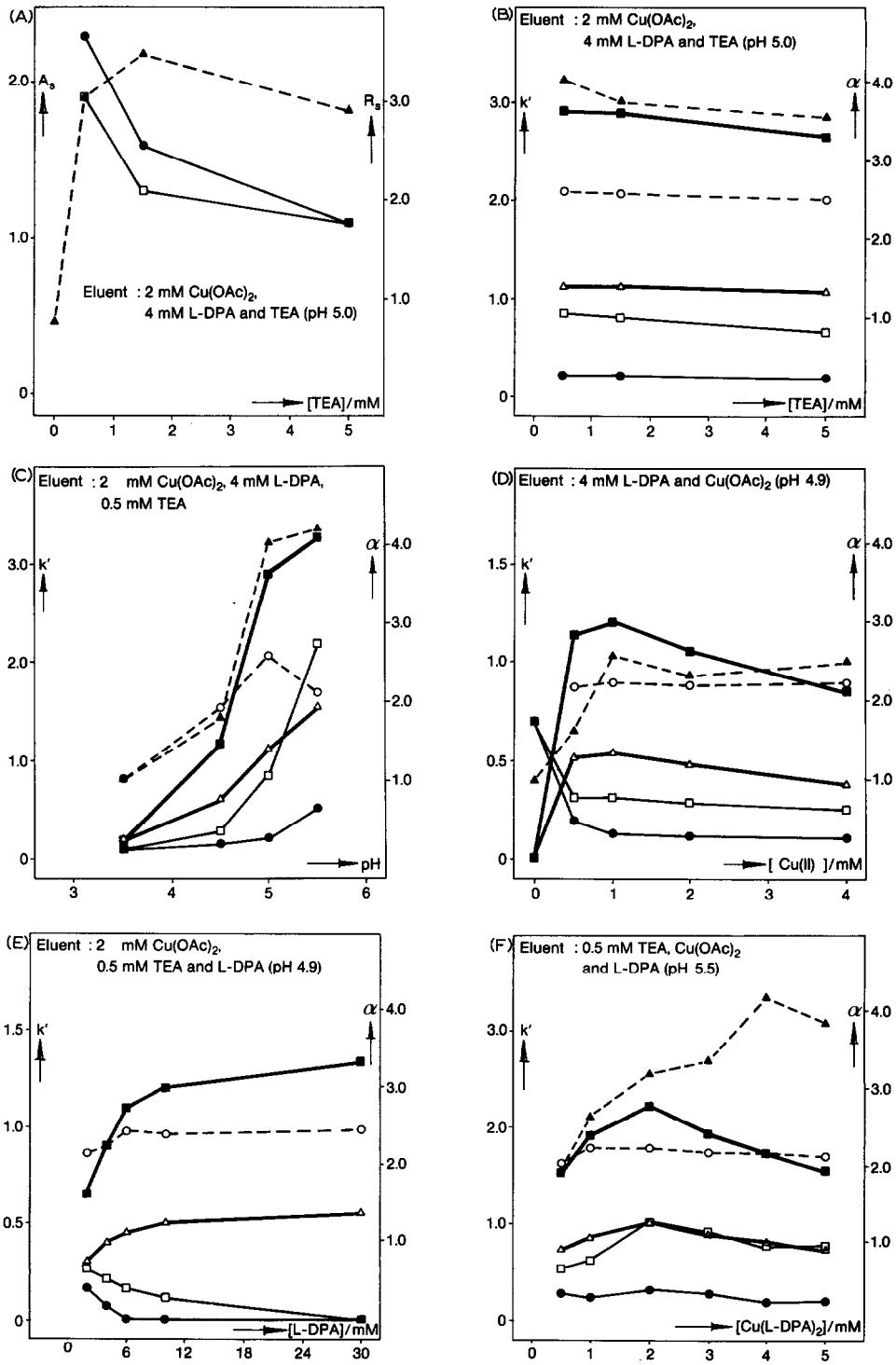
(A)
A_s
R_s
Eluent : 2 mM $Cu(OAc)_2$,
4 mM L-DPA and TEA (pH 5.0)
[TEA]/mM
(B)
Eluent : 2 mM $Cu(OAc)_2$,
4 mM L-DPA and TEA (pH 5.0)
k'
α
[TEA]/mM
(C)
Eluent : 2 mM $Cu(OAc)_2$, 4 mM L-DPA,
0.5 mM TEA
k'
α
pH
(D)
Eluent : 4 mM L-DPA and $Cu(OAc)_2$ (pH 4.9)
k'
α
[Cu(II)]/mM
(E)
Eluent : 2 mM $Cu(OAc)_2$,
0.5 mM TEA and L-DPA (pH 4.9)
k'
α
[L-DPA]/mM
(F)
Eluent : 0.5 mM TEA, $Cu(OAc)_2$
and L-DPA (pH 5.5)
k'
α
$[Cu(L\text{-}DPA)_2]$/mM

Fig. 1.

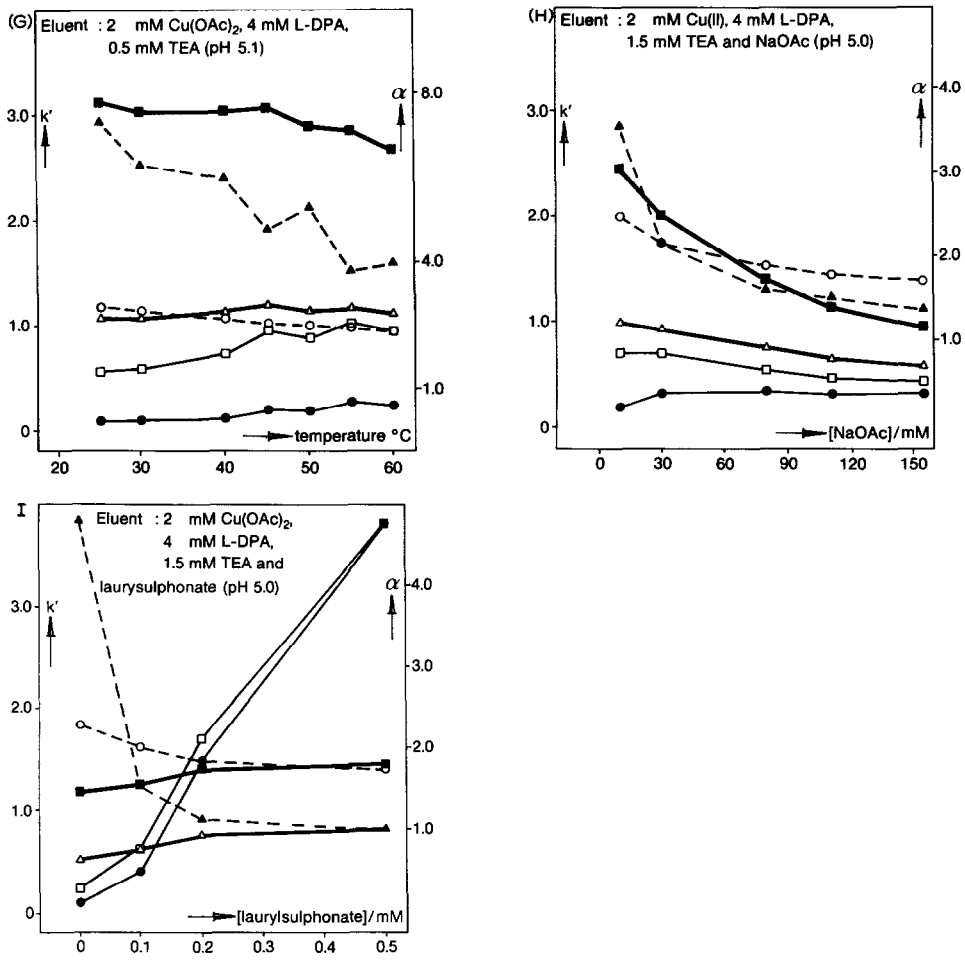

Fig. 1. Effect of (A, B) triethylamine concentration, (C) eluent pH, concentrations of (D) Cu^{II}, (E) L-DPA and (F) Cu(L-DPA)$_2$, (G) column temperature, (H) ionic strength and (I) laurylsulphonate concentration on the retention and selectivity of Val and Val-NH_2 enantiomers. Columns: (A, B, C, G, H) Hypersil ODS and (D, E, F, I) Polygosil 60-C_{18}. For other conditions, see Experimental. k': △, D-Val; ■, L-Val; ●, D-Val-NH_2; □, L-Val-NH_2. α: ○, D,L-Val; ▲, D,L-Val-NH_2.

effect is observed for the α-value. For the Val-NH_2 enantiomers, the retention and selectivity also increase with increasing pH. However, the increase is much steeper than with Val. Above pH 5, the elution order of L-Val-NH_2 and D-Val is reversed. The increase in k' and α of the compounds studied can be explained as follows: decreasing the acidity of the eluent favours deprotonation of the ammonium group of Val and Val-NH_2 and thus enhances the formation of mixed-ligand copper complexes, which are assumed to be primarily responsible for both the retention and enantioselectivity. The marked increase in the k' value of Val-NH_2 compared with Val can be explained in terms of the basicity of these compounds. The α-NH_2-function of Val-NH_2 is less basic than that of Val (Δ p$K_a \approx 2$).

Starting from the ammonium form of Val and Val-NH_2 at pH 3.5, an increase in the eluent pH will first generate the unprotonated α-NH_2 of Val-NH_2 and only after a further increase in the pH will the α-NH_2 function of Val be deprotonated and thus be able to form mixed-ligand copper complexes. From this study, it is clear that the eluent pH is a powerful device for tailoring the resolution of the enantiomers of an α-AA and the corresponding α-AA-NH_2.

Effect of Cu^{II}

At a constant concentration of L-DPA, the influence of the Cu^{II} concentration in the eluent on the k' and α values of the enantiomers of Val and Val-NH_2 was studied (Fig. 1D). In the absence of Cu^{II}, no retention was obtained for Val. On addition of 0.5 m*M* Cu^{II}, retention occurred for the Val enantiomers. At Cu^{II} concentrations above 1 m*M*, a slight decrease in k' for both Val enantiomers was seen. However, the enantioselectivity remained constant at Cu^{II} concentrations above 1 m*M*.

The effect of the Cu^{II} concentration on the retention and enantioselectivity of Val-NH_2 is remarkable. In the absence of Cu^{II}, a higher k' for the enantiomers is obtained than in the presence of Cu^{II}. An increase in the Cu^{II} concentration results in a further decrease in the retention of the Val-NH_2 enantiomers. The increase in k' and α of the Val enantiomers in the Cu^{II} concentration range 0–1 m*M* is caused by the formation of Cu^{II}–L-DPA complexes, which will act as exchange sites for the enantiomers for the formation of mixed-ligand complexes.

An explanation for the decrease of the k' values of the Val-NH_2 enantiomers in the Cu^{II} concentration range 0–1 m*M* may be as follows: in the absence of Cu^{II}, the interaction of the hydrophobic part of the Val-NH_2 molecules with the reversed-phase matrix will produce retention. However, the tailing peak of Val-NH_2, in the absence of a competitive base, points to another interaction with the stationary phase, namely the interaction of the polar acid amide groups with the free silanol sites in the matrix. This interaction will give an additional increase in retention. In the presence of Cu^{II}, mixed-ligand complexes of Val-NH_2 with Cu^{II}–L-DPA will be formed. In the resulting complex the polar acid amide group will be coordinated with Cu^{II} and thus be unavailable to undergo interaction with the free silanol sites in the matrix. The absence of the latter interaction may explain the observed decrease in retention when the Cu^{II}–L-DPA system is used.

The addition of copper(II) acetate to the mobile phase will cause an increase in ionic strength, which may explain the slight decrease in the retention of both Val and Val-NH_2 enantiomers at Cu^{II} concentrations above 1 m*M*.

Effect of L-DPA

The influence of the L-DPA concentration in the eluent at a constant Cu^{II} concentration on the k' and α values of the enantiomers of Val and Val-NH_2 was studied (Fig. 1E). An increase in the L-DPA concentration results in an increase in the α and k' values of the Val enantiomers.

For the acid amide enantiomers, an opposite effect can be seen. Increasing the L-DPA concentration leads to a decrease in the k' values of the Val-NH_2 enantiomers and a diminution of enantioselectivity. The equilibrium reactions for the formation of the binary Cu (L-DPA)$_2$ complex can be written as

$$Cu^{2+} + \text{L-DPA}^- \rightleftharpoons [Cu(\text{L-DPA})]^+ \quad (1)$$

$$[Cu(\text{L-DPA})]^+ + \text{L-DPA}^- \rightleftharpoons [Cu(\text{L-DPA})_2] \quad (2)$$

These equilibria will be influenced by the pH of the eluent, which will determine the ionization state of the L-DPA (anion, zwitterion or cation) and by the Cu^{II}:L-DPA concentration ratio. Considering the starting conditions in Fig. 1E (2 m*M* Cu^{II}, 2 m*M* L-DPA, pH 4.9), both $[Cu(\text{L-DPA})]^+$ and $[Cu(\text{L-DPA})_2]$ will be present in the eluent. On introduction of the α-AA and α-AA-NH_2 enantiomers into the eluent, two reactions can lead to mixed-ligand complex formation. For example, for the L-form of the α-AA these reactions can be written as

$$[Cu(\text{L-DPA})_2] + \text{L-AA}^- \rightleftharpoons [(\text{L-AA})Cu\ (\text{L-DPA})] + \text{L-DPA}^- \quad (3)$$

$$[Cu(\text{L-DPA})]^+ + \text{L-AA}^- \rightleftharpoons [(\text{L-AA})Cu\ (\text{L-DPA})] \quad (4)$$

Only in reaction 3 does ligand exchange take place. In reaction 4 mixed-ligand complexes are formed without exchange. Increasing the L-DPA concentration in the eluent at a constant Cu^{II} concentration will shift the equilibria of reactions 1 and 2 to the right, resulting in a higher concentration of $Cu(\text{L-DPA})_2$ complexes. This implies that more interaction sites become available for the enantiomers to form mixed-ligand complexes.

The increase in the *k'* values for the Val enantiomers can therefore be explained in terms of an increase in mixed (L-Val)Cu(L-DPA) and (D-Val)Cu(L-DPA) complexes formed by ligand exchange. For the Val-NH_2 enantiomers it is unlikely that the formation of mixed-ligand complexes will proceed via ligand exchange. This can be seen by comparing tabulated equilibrium constants[10] for the reaction

$$Cu^{II} + L \rightleftharpoons Cu^{II}L \quad (5)$$

With Gly–Gly-NH_2 the highest equilibrium constant is found for the α-AA. This indicates that the coordination of an α-AA to Cu^{II} is much stronger than that of an α-AA-NH_2. Therefore, it is very doubtful that an α-AA-NH_2 (*e.g.*, Val-NH_2) will displace an α-AA (*e.g.*, L-DPA) from Cu^{II}. The data we found for the Val-NH_2 enantiomers (Fig. 1E) can be explained on the basis of reaction 4. Increasing the L-DPA concentration in the eluent will lead to an increase in $Cu(\text{L-DPA})_2$ complexes and a decrease in $[Cu(\text{L-DPA})]^+$. This decrease means that the equilibrium of reaction 4 is shifted to the left, resulting in fewer $[(\text{L-Val-NH}_2)Cu(\text{L-DPA})]^+$ and $[(\text{D-Val-NH}_2)Cu(\text{L-DPA})]^+$ complexes. This in turn results in a decrease in the *k'* values of the Val-NH_2 enantiomers (Fig. 1E). The formation of mixed-ligand complexes involving α-Val-NH_2 may therefore proceed preferentially under the conditions where the L-DPA:Cu^{II} concentration ratio is 1 rather than at higher ratios. For α-hydroxy acids it has also been suggested that a 1:1 ratio of Cu^{II} and L-Pro (as chiral selector) is important for the formation of mixed-ligand complexes[7].

Effect of Cu(L-DPA)$_2$ concentration

The influence of the $Cu(\text{L-DPA})_2$ concentration in the eluent on the *k'* and α values of Val and Val-NH_2 enantiomers is presented in Fig. 1F. From 0.5 to 2 m*M*

Cu(L-DPA)$_2$, the k' values for both Val and Val-NH$_2$ enantiomers increase, whereas above 2 m*M* Cu(L-DPA)$_2$ a decrease in the k' values of all enantiomers can be seen. The α value for the Val enantiomers slowly decreases, whereas for the Val-NH$_2$ enantiomers the increase in α continues at Cu(L-DPA)$_2$ concentrations above 2 m*M*. If the Cu(L-DPA)$_2$ concentration in the eluent is lower than the concentration at which the stationary phase is saturated, retention and selectivity will be determined by the amount of [Cu(L-DPA)$_2$] and [Cu(L-DPA)]$^+$ adsorbed on the stationary phase. The behaviour of k' and α in the range 0.5–2 m*M* Cu(L-DPA)$_2$ therefore seems to originate from the loading of the stationary phase with the chiral complexes. Once the stationary phase is saturated with [Cu(L-DPA)$_2$] and [Cu(L-DPA)]$^+$, further addition of Cu(L-DPA)$_2$ will increase its concentration in the mobile phase. The enhancement of the electrolyte concentration probably accounts for the decrease in retention observed at Cu(L-DPA)$_2$ concentrations above 2 m*M*.

The two *n*-propyl chains of L-DPA may play an important role in the observed behaviour of the enantiomers studied, as a similar mechanism for the retention and enantioselectivity has been proposed for other chiral ligands with long alkyl side-chains[8,11–13].

Effect of column temperature

As the equilibria in the present system should be temperature-dependent, the effect of column temperature was studied. In Fig. 1G, the influence of temperature on the k' and α values of Val and Val-NH$_2$ enantiomers is given. For L-Val a decrease in k' can be seen, whereas the k' of D-Val remains nearly constant on raising the column temperature. The enantioselectivity for Val decreases. For the Val-NH$_2$ enantiomers the k' values increase on increasing the temperature. The latter effect could be explained by a shift of equilibrium 4 towards the mixed-ligand complex. However, in this instance one would expect an increase in the enantioselectivity of Val-NH$_2$; however, this is not observed (Fig. 1G). The explanation of the increase in the k' values of Val-NH$_2$ enantiomers, together with the decrease in enantioselectivity, is therefore not clear to us.

Effect of ionic strength

To investigate the importance of electrostatic effects, the influence of ionic strength on the k' and α values of Val and Val-NH$_2$ enantiomers was studied (Fig. 1H). The ionic strength of the eluent was regulated by means of sodium acetate. Over the concentration range studied the k' and α values of Val and Val-NH$_2$ enantiomers decreased. The decrease was most noticeable at low concentrations of acetate, where the drop in k' and α is fairly rapid. At higher concentrations the change in k' and α is much more moderate.

Effect of alkylsulphonate

Aliphatic α-AA and α-AA-NH$_2$ with small hydrophobic side-chains (*e.g.*, Ala–Ala-NH$_2$) had low k' values in the chromatographic system employed. In order to increase the retention of these compounds, the influence of an ion-pairing reagent in the eluent on the k' and α values of the enantiomers of Val and Val-NH$_2$ was studied (Fig. 1I). Laurylsulphonate was chosen for ion-pair formation.

For the Val enantiomers, a moderate increase in the k' values was obtained over

the range 0–0.5 m*M*. The enantioselectivity decreased over this range. The Val-NH_2 enantiomers showed a steep increase in k' at increasing sulphonate concentration, while a rapid drop in enantioselectivity occurred, resulting in a loss of selectivity at a concentration of 0.5 m*M*. The increase in retention and the decrease in enantioselectivity can be explained by assuming a competition between mixed-ligand formation and ion-pair formation. Because the coordination of an α-AA-NH_2 to Cu^{II} is much weaker than that of an α-AA[10], the effect of the ion-pairing reagent is greatest on Val-NH_2 (Fig. 1I). This study indicates that the use of an ion-pairing reagent may be a device for enhancing the retention of enantiomers with low k' values; however, its use is limited to some extent as the enantioselectivity decreases. Another important aspect of the use of an ion-pairing reagent is the fact that it is a powerful device for discriminating between an α-AA and α-AA-NH_2. If enantiomeric reolution of an α-AA is vitiated by overlap with the corresponding α-AA-NH_2, the use of an ion-pairing reagent may be useful in eliminating the interference effect.

Chromatography

The feasibility of the separation system was tested for DL-β-methyl-Ala, DL-Met, DL-α-phenyl-Gly, DL-Phe, DL-β-benzyl-Ala and their corresponding amides. For all compounds tested a separation of the amino acid and the amide enantiomers could be achieved. The α-AA-NH_2 enantiomers were eluted before the α-AA enantiomers and in all instances the D-enantiomer was eluted before the L-enantiomer. With the hydrophobic β-benzyl-Ala and β-benzyl-Ala-NH_2, acetonitrile was added to the mobile phase to shorten the retention times. A representative chromatogram of a sample from a process stream is given in Fig. 2.

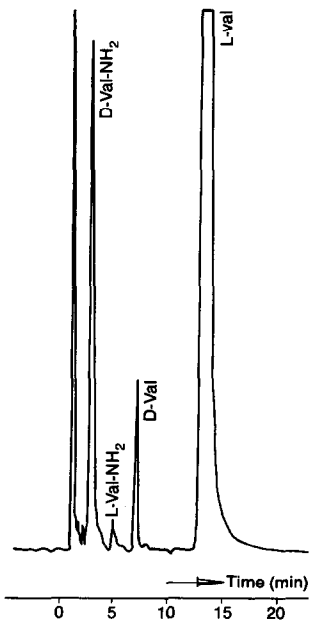

Fig. 2. Chromatogram of a sample from L-Val synthesis. Column: Nucleosil 120-5C_{18}. Mobile phase: 2 m*M* copper(II) acetate–4 m*M* L-DPA–1 m*M* TEA (pH 5.1). For other conditions, see Experimental.

Quantitative determinations were carried out by comparing the peak areas of samples with those of standard solutions, employing the external standard method. As an example of the linearity, precision and detection limit of the method, these data are given for Val and Val-NH_2. The linearity of the amount *versus* response relationship was established over the ranges 3–40 nmol (DL-Val) and 10–75 nmol (DL-Val-NH_2). Linear regression analysis from calibration graphs indicated that the correlation coefficients for the enantiomers were 0.9997 (Val) and 0.9998 (Val-NH_2). The within-run precision of the assay gave a coefficient of variation of $\leqslant 2\%$ for both Val and Val-NH_2. The detection limits, calculated as two times the noise ratio, and expressed in terms of picomoles of the compounds injected, were 20 and 400 for Val and Val-NH_2, respectively.

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