HPTLC Separation of Aromatic α-Amino Acid Enantiomers on a New Histidine-Based Stationary Phase Using Ligand Exchange

M. Remelli / R. Piazza / F. Pulidori*

Dipartimento di Chimica, Università di Ferrara, via Luigi Borsari 46, 44100 Ferrara, Italy

Key Words

Thin-layer chromatography
Chiral separation
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Summary

A new chiral ligand exchange selector for hydrophobic stationary phase modification has been synthesized by selective alkylation of L-histidine at the pyrrolic nitrogen atom in its imidazolic ring. Its performance was then tested on RP-HPTLC plates treated with copper acetate. Significant selectivity towards aromatic amino acid enantiomers, was observed. Chromatographic retention data were compared to available thermodynamic complex formation parameters for the relevant model systems in aqueous solution.

Stationary and mobile phase effects on retention were studied by using different RP-HPTLC plates and various binary aqueous solvent mixtures.

Optimized separation conditions for aromatic amino acid sample class are given.

Introduction

The development of new techniques for the resolution of racemic mixtures has been receiving increasing attention, especially in biological and pharmacological fields. It is well known that some optical antipodes can exert very different effects on living organisms. Many of the most efficient chiral separation methods are based on the chromatographic mechanism: a number of books and reviews have recently been published on this topic [1–7]. In this context, particular interest is focused on amino acid enantiomer resolution, both on the analytical and preparative scale, and various separation methods have been reported. These can generally be classified as: those using (1) chiral stationary phases; (2) the addition of a chiral reagent to the mobile phase with the formation of diastereomeric adducts separable on a conventional non-chiral stationary phase; (3) precolumn derivatization of the analytes, again to give diastereomeric isomers.

Success has been achieved in underivatized amino acid enantiomer separation via chiral ligand exchange chromatography (CLEC) by using a chiral mobile or stationary phase [8]. This latter technique, first introduced in chiral separation by Rogozhin and Davankov [9], is based on the formation of labile ternary metal (generally copper(II)) complexes in the stationary phase: one of the ligands is an appropriate chiral selector chemically bonded to, or permanently adsorbed on, a solid support, while the second ligand is one of the components of the sample mixture. The stationary phase is loaded with the complexing metal ion, low concentrations of which are generally present in the mobile phase as well. In this manner, enantiomeric mobile ligands form stationary diastereomeric complexes: even small differences in stability between these complexes can lead to enantiomer separation. To date, various chiral selectors have been used in CLEC. They are often composed of an appropriate L-amino acid derivative, particularly an L-proline derivative [10–13].

Potentiometric [14, 15] and calorimetric [15] studies in aqueous solution have revealed significant thermodynamic stereoselectivity between ternary mixed copper complexes of L- or D-histidine and a second L-amino acid. In particular, when the latter is an amino acid bearing an aromatic side chain (L-tryptophan, L-phenylalanine), the *hetero-chiral* complex (containing ligands of different chirality) is more stable than the *homo-chiral* complex (in which both the ligands have the same chirality). The differences in free energies of formation are approximately 1–2 kJ mol^{-1} while the differences in formation enthalpies are approximately 2–6 kJ mol^{-1}. Smaller but significant stereoselective effects were also found in the formation of ternary Cu(II) complexes of L- or D-histidine with aliphatic amino acids; however, in this case, the *homo-chiral* complex formation is favoured. Moreover, thermodynamic and spectroscopic studies have shown that these ternary complexes are nearly octahedral: histidine behaves as a tridentate ligand, binding the Cu(II) ion with its amino and pyridino nitrogens in equatorial positions and with a carboxylate oxygen in a distorted axial position. This behaviour is the basis for the stereoselectivity found (see Figure 1).

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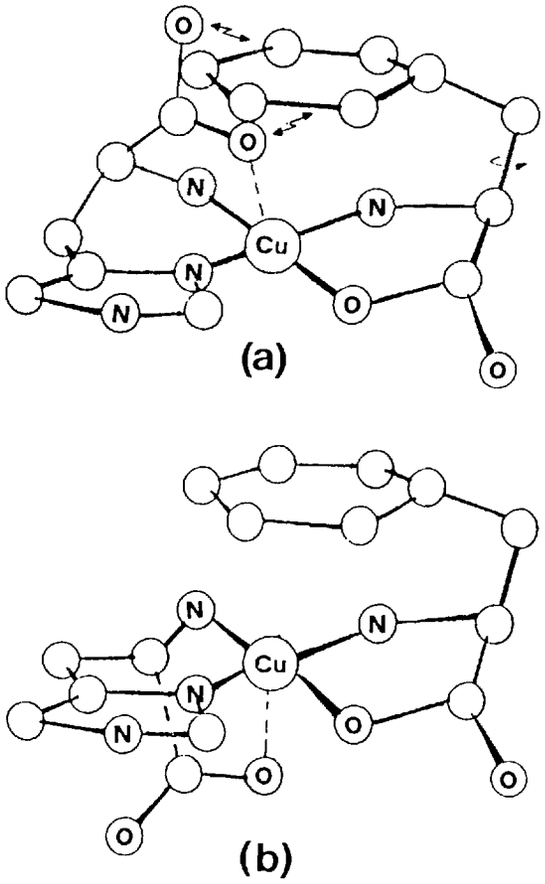

Figure 1

Copper(II) co-ordination mode in ternary complexes with L-histidine (*a*) or D-histidine (*b*) and L-phenylalanine, and possible disposition of side-chain residues (from ref. [15]).

The above results suggest that histidine, if properly immobilized on a solid support, can act as an efficient chiral selector for CLEC. Moreover, if adequate experimental conditions are selected, chromatographic results could be interpreted in the light of thermodynamic solution data, although one must bear in mind that there are substantial differences between the two environments where the mixed complexes are formed. For this comparison to be reliable, it is essential to preserve the above-mentioned histidine complexing properties when planning the synthesis of the histidine based chiral selector; this is achieved by keeping the functional groups involved in copper ion binding free. Some histidine derivatives were previously employed as chiral selectors in CLEC either as physically adsorbed [16] or chemically bonded [17, 18] modifiers in the stationary phase. However, they were synthesized by reaction at the α-amino or α-carboxylic group.

Despite the popularity of thin layer chromatography (TLC) due to its simplicity, low cost, wide versatility and ability to screen a large number of samples in a single run, relatively few applications have been reported in the literature on TLC or HPTLC separation of racemic mixtures with chiral stationary phases [19–21]. One of the most successful methods in this field is again the one based on the CLEC separation mechanism, generally using a stationary phase impregnated with a suitable chiral ligand complexed with copper ions. Weinstein [22] covered reversed phase plates with N,N-di-n-propyl-L-alanine and cupric acetate on which various dansyl-amino acid enantiomers were separated; subsequently, Grinberg and Weinstein [23] improved this method developing a two dimensional RP-TLC technique. Marchelli et al. [24] also separated dansyl-amino acid enantiomers on pre-coated HPTLC RP-18 plates impregnated with copper acetate and a chiral selector consisting of two amino acids bonded to an alkyl diamine through the formation of amido bonds. Günther et al. [12, 25–27] described a chiral phase made by treating octadecyl-modified TLC plates with a solution of copper acetate followed by a solution of (2S,4R,2'RS)-4-hydroxy-1-(2'-hydroxydodecyl)proline. These plates are now commercially available as Chiralplates from Macherey-Nagel (Düren, FRG, cat. n. 811055/056) and a certain number of racemic samples have been resolved on them: several types of amino acids (proteinogenic, non-proteinogenic, N-alkyl and halogenic), dipeptides, α-hydroxycarboxylic acids, some heterocyclic compounds [12, 25–28], N-carbamyl-trypthophan [29], purine nucleosides [30], phenylalanine and tyrosine derivatives [31], N-methylaspartic acid enantiomers [32]. Moreover, a paper has been published on the use of Chiralplates in various types of forced-flow planar chromatographic techniques [33]. Finally, a new type of chiral HPTLC plate has recently been developed by Merck (Darmstadt, FRG) and is commercially available under the trade name CHIR (Merck, art. n. 14285). It is based on a reversed-phase matrix impregnated with a copper salt and an optically active amino acid. These plates, supplied with a concentrating zone, proved to be suitable for amino acid and α-hydroxycarboxylic acid enantiomer separation [13].

The present work describes the preparation and use of new chiral plates for CLEC. The chiral selector employed here is the newly synthesized L-N^{τ}-n-decylhistidine (LNDH), the n-decyl derivative of L-histidine selectively alkylated at the pyrrolic nitrogen atom of its imidazolic ring (see Figure 2). The separation of racemic mixtures of underivatized amino acids has been studied under different experimental conditions.

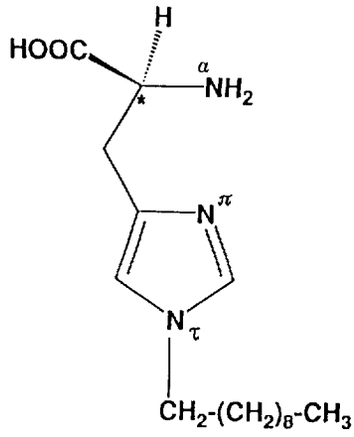

Figure 2

Structure of chiral selector L-N^{τ}-n-decylhistidine.

Experimental

Synthesis and Characterization of Chiral Selector

The chiral selector, L-N^{τ}-n-decylhistidine (LNDH) was synthesized following the procedure by Noordam et al. [34], who developed the regioselective synthesis of the corresponding methyl and ethyl derivative. The first reaction step was the temporary protection of α and π nitrogen atoms of L-histidine (taken as methyl ester) by reaction with N,N'-carbonyldiimidazole. The product was then alkylated at its τ nitrogen atom by reaction with 1-iododecane. Finally, acidic hydrolysis and subsequent careful titration with sodium hydroxide up to a slightly alkaline pH, gave LNDH as free base.

A mixture of L-histidine methyl ester dihydrochloride (12.1 g, 50 mmol) and N,N'-carbonyldiimidazole (10 g, 60 mmol) in 250 ml anhydrous N,N-dimethylformamide (DMF) was stirred at 65 °C. After stirring for 6 h the DMF was removed *in vacuo*, yielding an oil, which was dissolved in 500 ml of saturated aqueous $NaHCO_3$. The aqueous solution was then extracted fifteen times with 80 ml portions of chloroform. The combined chloroform layers were dried over $MgSO_4$; after filtration the chloroform was removed *in vacuo* yielding a solid (I) which was crystallized from cold ethyl ether (yield: 6.9 g, 35 mmol, 75 %).

To a solution of (I) in 100 ml anhydrous DMF (6.9 g, 35 mmol) was added a double amount (19 g, 70 mmol) of 1-iododecane. The solution was stirred at room temperature for ten days; after three and six days, other 9.5 g (35 mmol) of 1-iododecane were added. The DMF was then removed *in vacuo*, yielding an oil, which was washed twenty times with 50 ml portions of ethyl ether, thus obtaining a crystalline powder (II) (yield: 12.4 g, 26.7 mmol, 76 %).

A solution of 12 g (26 mmol) of product (II) in 100 ml M HCl, containing a minimum of dioxane, was refluxed for 14 h. The solution was evaporated to about 1/3 its initial volume and titrated very slowly with M NaOH, in an ice bath with stirring, until the complete precipitation of LNDH as free base was reached. After filtration, LNDH was dissolved in the minimum volume of methanol and crystallized from water/methanol (yield: 5.6 g, 17.2 mmol, 66 %).

An analytical sample of LNDH was fully characterized:
m.p. 215–220 °C (dec.);
$[\alpha]_D^{20} = -9.5°$ (*c* 1.3, methanol);
$C_{16}H_{29}O_2N_3$ (m.w. = 295.43), calcd C 65.1 %, H 10.8 %, N 14.2 %, found C 64.5 %, H 10.3 %, N 13.9 %;
^{1}H-NMR (CD_3OD): δ (ppm) 0.9 (3H, t: J = 6.6 Hz, CH_3), 1.3–1.4 (14H, m, $CH_3(CH_2)_7$), 1.8 (2H, m, $CH_3(CH_2)_7CH_2$), 3.15 (2H, ABX: J_{AX} = 9.9 Hz, J_{BX} = 3.7 Hz, J_{AB} = 15.3 Hz, $COOH(NH_2)CHCH_2$), 3.85 (1H, d.d.: J_{AX} = 10.0 Hz, J_{BX} = 3.7 Hz, $COOH(NH_2)CH$), 4.0 (2H, t: J = 7.0 Hz, $CH_3(CH_2)_7CH_2CH_2$), 7.0 (1H, s, CCHN), 7.6 (1H, s, NCHN);
^{13}C-NMR (CD_3OD) δ (ppm): 14.46 (CH_3), 23.73 (CH_3CH_2), 32.06, 33.05 ($COOH(NH_2)CHCH_2$, $CH_3CH_2(CH_2)_7CH_2$), 56.40 ($COOH(NH_2)CH$), 118,64 (CCHN), 137.58 (NCCHN), 138.34 (NCHN), 173.86 (COOH);
TG/DTG: thermally stable up to 215 °C, without weight loss;
DSC: exothermic peak (in air) at 210.1 °C.

The enantiomeric purity of the product was checked via ^{1}H-NMR using the chiral shift reagent tris[3-(epta-fluoro-propylhydroxymethylene)-(+)-camphorate]europium(III).

Chiral Plate Preparation

The followed procedure was suggested by Günther et al. [12]. RP-18 $WF_{254}S$ HPTLC plates (Merck art. n. 13124) were immersed for about 5 minutes in a solution of Cu(II) acetate (Riedel-De Haën, ACS) 0.125 % w/v in water/methanol (90:10). After drying at room temperature, the plates were immersed for 1 minute in 0.4 % w/v methanolic LNDH. The plates were then dried at room temperature and were ready for use or could be stored for later use.

Chromatographic Conditions

The amino acids were high purity products (Aldrich and Serva) and the solvents were HPLC grade (Carlo Erba). Amino acids, in a water/methanol solution (≈ 0.1 % w/v), were applied to the plates with a Linomat IV (Camag, Mutenz) equipped with a 100 µl microsyringe (Hamilton); sample volume: 1–3 µl; delivery rate: 4 sec µl^{-1}. The samples were developed in an unsaturated chamber using various aqueous solvents. After drying at room temperature, the plates were dipped for few seconds in 0.15 % w/v ninhydrin solution in acetone and then oven dried at 110 °C for about 3 min. Intense red or violet spots developed on a lighter red field.

Results and Discussion

Different siliceous C_{18} plates could be used as solid support for modification with LNDH. The surface of W type Merck plates chosen for this investigation is only partially covered with C_{18} hydrocarbon chains and, consequently, they show lower hydrophobicity than a completely modified RP-18 support [35]. In view of the presence of a certain amount of free silanols, this feature makes it possible to use aqueous solvents: a) as mobile phase; b) for the dipping solutions employed in stationary phase preparation and c) for sample solutions. Homogeneous coverage with the chiral selector was obtained through the method previously proposed by Günther et al. [12] (see experimental section). Reversing the order of the dipping solutions or performing a unique dipping in a solution already containing the preformed Cu(II)/LNDH complex did not yield homogeneous coverage.

Racemic mixtures of aliphatic (alanine, Ala; valine, Val; leucine, Leu) and aromatic (phenylalanine, Phe; tryptophan, Trp) amino acids in hydroalcoholic solution were first used as samples to be separated. An initial task of this work was to verify whether the thermodynamic stereoselectivity found between *hetero-chiral* and *homo-chiral* Cu(II) complexes of these ligands with histidine (His) in aqueous solution, could lead to significant enantioseparation of these amino acids in the chromatographic system. The solvent used was that suggested by Günther et al. [12]; that is methanol(MeOH)/water/acetonitrile(ACN) 50:50:200.

Good results have been found in the case of aromatic amino acids, while for aliphatic samples (e.g. DL-Val, DL-Ala and DL-Leu) no appreciable separation could be observed. As far as the aromatic samples are concerned, the D-enantiomer was the most retained; R_m (= log[1/R_f – 1]) values showed the same trend as the corresponding free energies of formation for the ternary *hetero-chiral* and *homo-chiral* complexes [Cu(II)(His)(Trp)] and [Cu(II)(His)(Phe)] in aqueous solution [15]. Such re-sults strongly suggest that the ligand-exchange mechanism controls enantiomer separation, while the mean retention shown by each enantiomeric pair most likely depends chiefly on the hydrophobic properties of the side chain as well as on the eluent composition. In Table I the R_m and α (= $R_{f(L)}/R_{f(D)}$) values for D/L-Trp and D/L-Phe samples are shown, along with the corresponding data reported by Günther et al. and the results obtained with commercial chiral plates CHIR (Merck), with the same eluent composition. A better resolution was obtained for these analytes. On the other hand, the inability to separate aliphatic amino acids as well is probably due to the intrinsically poor efficiency of the TLC system together with the fact that, because of problems inherent in detection, it proved impossible to increase chiral selector surface concentration. In fact, even the His-based selector (LNDH) covering the stationary phase reacts with ninhydrin developing a red colour, the intensity of which depends on the stationary phase preparation method and on the selector concentration at the surface. When the degree of coverage was too high, sample spot detection became practically impossible; when it was too low chiral separation efficiency was poor. A compromise was found by varying the concentration of the LNDH solution used as dipping reagent as well as the dipping time. The best conditions are those reported in the experimental section; no improvement was reached changing the developing reagent to potassium permanganate or using the iodine chamber. However, it is worth noting that the aliphatic amino acid spots obtained after development appeared generally abnormally elongated, but not tailed.

In order to get more information on solvent selectivities as well as on the separation mechanism, a systematic study was carried out employing various binary aqueous solvents. The L/D-Trp pair was selected for this purpose. The organic solvents considered here are those commonly used in reversed phase TLC; that is, MeOH, ethanol (EtOH), isopropanol (i-PrOH), ACN, acetone (DMK) and tetra-hydrofuran (THF). The results are reported in Figure 3.

Regular RP behaviour was obtained with alcohols as well as with THF. In fact, the R_m values are expected to decrease with increasing solvent strength: that is, increasing the percentage of the organic component. At the same time the corresponding α values decrease, due to the levelling effect of a stronger solvent. Conversely, when the solvent in the mixture was ACN or DMK a minimum in the R_m curve was found when the organic solvent content was approximately 60–70 %. In particular, for ACN, the second half of the curve is nearly a straight line with a positive slope. Similar behaviour was also found for Phe and Val, under analogous conditions. Moreover, the corresponding α values show an initial decrease and then they remain just about constant even at high organic solvent concentrations. Very similar R_m trends were also found with commercial Merck CHIR plates; as far as the α values are concerned, they increase slightly with increasing solvent strength (see Figure 4).

Full explanation of the above results is not easy: various retention mechanisms contribute simultaneously to overall retention. Among them, solvophobic and silanophilic interactions, specific solvation of the solute and the stationary phase as well as ligand-exchange equilibria play an important role. While the latter is responsible for enantiomer separation, it is difficult to decide whether the "dual slope" behaviour observed in the R_m vs. ACN (DMK) % curves is exclusively due to a solvent effect or whether it is affected by the overall solute-stationary phase interactions. The strength of the organic solvent in the binary aqueous mixture is no doubt strongly influenced by sample solubility. It is well known that amino acids are somewhat soluble but only in water; Trp is sparingly soluble in hot alcohols while it is practically insoluble in DMK and in ACN. This suggests that the high R_m values found at high DMK or ACN percentages might be due to the difficulty these solvents have in dissolving and then transporting samples.

For deeper insight into the retention mechanism, it was advisable, to separate the different contributions from the various stationary phase components. Therefore, similar R_m-versus-ACN % curves were obtained for the L/D-Trp

Table I. Retention parameters of tryptophan and phenylalanine enantiomers on different chiral HPTLC plates. Solvent: MeOH/H_2O/ACN 50:50:200.

R_m(L-Trp)	R_m(D-Trp)	α*	Plate type	Reference
0.52	0.72	1.44	Merck RP-18 $WF_{254}S$/LNDH/Cu	This work
–0.19	–0.02	1.20	Chiralplate (Macherey-Nagel)	[12]
–0.52	–0.29	1.17	CHIR (Merck)	This work
R_m(L-Phe)	R_m(D-Phe)	α*	Plate type	Reference
0.25	0.43	1.33	Merck RP-18 $WF_{254}S$/LNDH/Cu	This work
–0.16	0.02	1.20	Chiralplate (Macherey-Nagel)	[12]
–0.41	–0.21	1.16	CHIR (Merck)	This work

* α = R_f(L-Trp)/R_f(D-Trp)

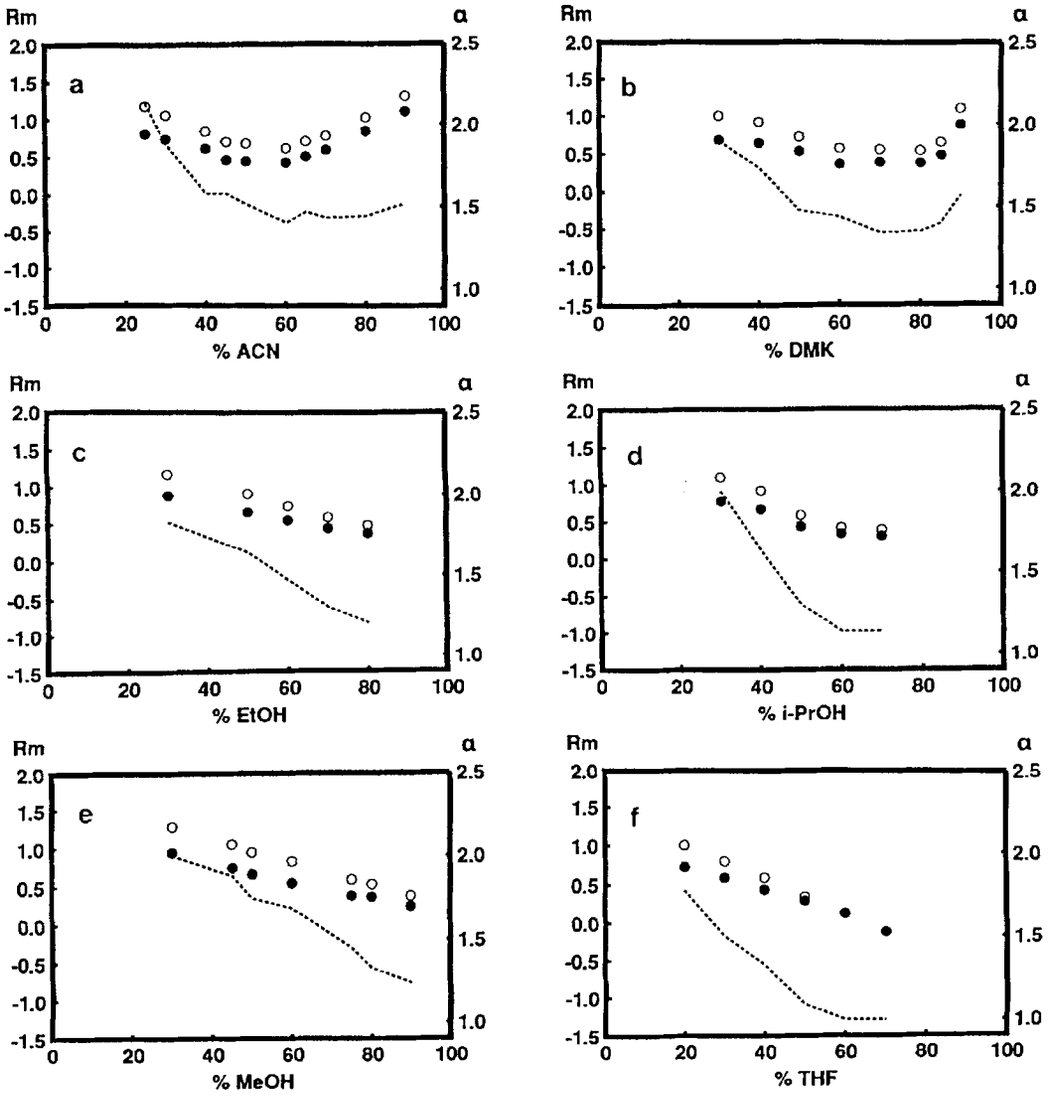

Figure 3

(a–f) Retention of L/D-Trp sample on RP-18 $WF_{254}S$ Merck plates covered with copper acetate and LNDH versus % organic solvent in aqueous binary eluents. Circles: R_m for D-Trp; bullets: R_m for L-Trp; dotted line: enantioselectity $\alpha = R_f(\text{L-Trp})/R_f(\text{D-Trp})$.

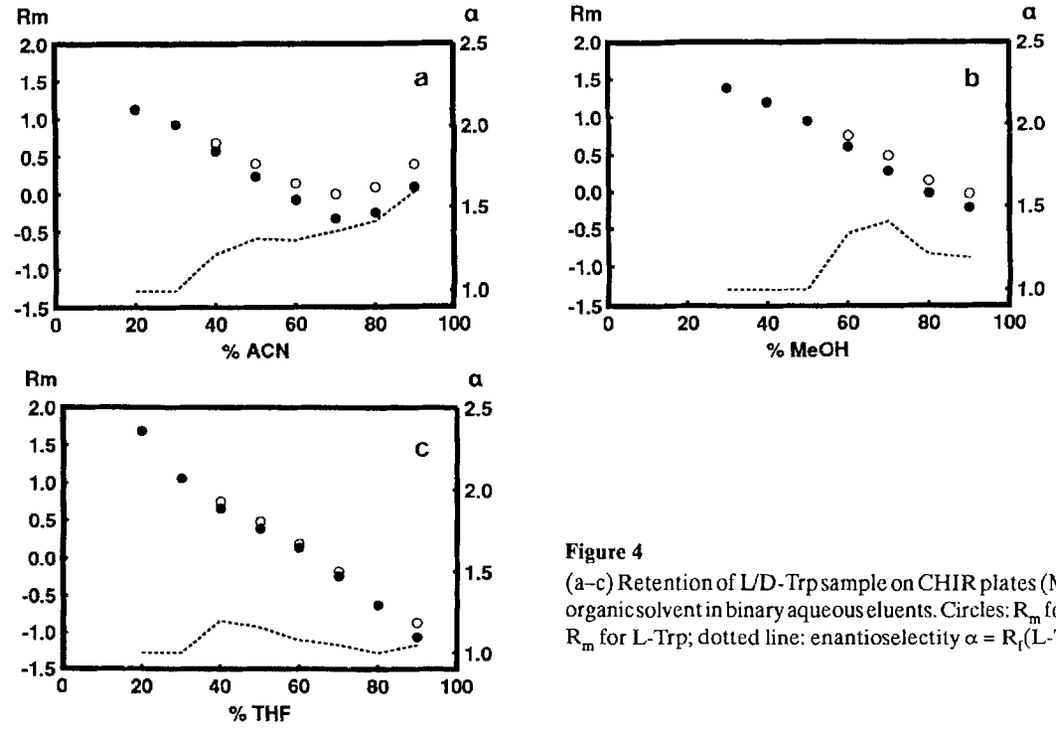

Figure 4

(a–c) Retention of L/D-Trp sample on CHIR plates (Merck) versus % organic solvent in binary aqueous eluents. Circles: R_m for D-Trp; bullets: R_m for L-Trp; dotted line: enantioselectity $\alpha = R_f(\text{L-Trp})/R_f(\text{D-Trp})$.

sample by using the same plates treated only with the copper acetate solution or without any treatment. No enantiomer separation was found in these cases, as expected; R_m values progressively decreased while eliminating stationary phase components (ternary stationary Cu(II) complexes containing LNDH and Trp are more stable than binary Cu(II)/Trp complexes, as in aqueous solution); the curves are quite similar to these found with Cu(II)/LNDH modified stationary phase. Therefore plate coverage does not play an important role in determining the "dual slope" behaviour of R_m-versus-ACN (DMK) % curves. Finally, a new set of experiments was performed with end-capped RP-18 plates (Merck, art. n. 5914), without any further treatment, although, in this case, the only experimentally accessible range was that of solvents with high ACN content. In this range, progressive increase in R_m values was found with increasing ACN. It can be concluded that neither the adsorbed chiral selector nor the Cu(II) ions, nor the free silanol sites contribute to form the observed U-shaped R_m curve. The above results are shown in Figure 5.

As a final step, a solvent optimization method was applied to the system to achieve the best separation conditions for aromatic amino acids and to attempt to separate aliphatic amino acids as well. The "PRISMA" method, developed by Nyiredy et al. [36], was followed. This method is oriented to the preparation of an optimized multi-component solvent, through a guided trial and error sequence. First, the basic parameters such as stationary phase and the individual solvents forming the eluent mixture have to be selected. On the basis of the R_m and α values obtained from the aqueous binary solvent curves discussed above (see Figure 3), MeOH, ACN and THF were chosen. Second, the optimum combination of these selected solvents is attained with the actual PRISMA model.

The first step concerned the total solvent strength adjustment, to achieve adequate R_f values (optimum R_f values range from 0.2 to 0.8). This was achieved by varying the total water content, with unitary ratios of the organic solvents. The second step was selectivity optimization. Keeping the total solvent strength constant, the proportion of individual organic components was appropriately varied. An optimized chromatogram is shown in Figure 6. The retention data for some aromatic amino acid enantiomers, under optimized elution conditions, are reported in Table II. R_m values, in particular for the D/L-Phe pair, are better than those obtained with the initial solvent (see Table I), while the resolution between enantiomers is still good. As far as the α-methylated amino acids are concerned, it can be observed that their retention is higher than that shown by the non-methylated samples, most likely due to the higher hydrophobicity of the whole molecule. Moreover, the enantioselectivity factor α is better in the former case. A good separation was also obtained for D/L-DOPA.

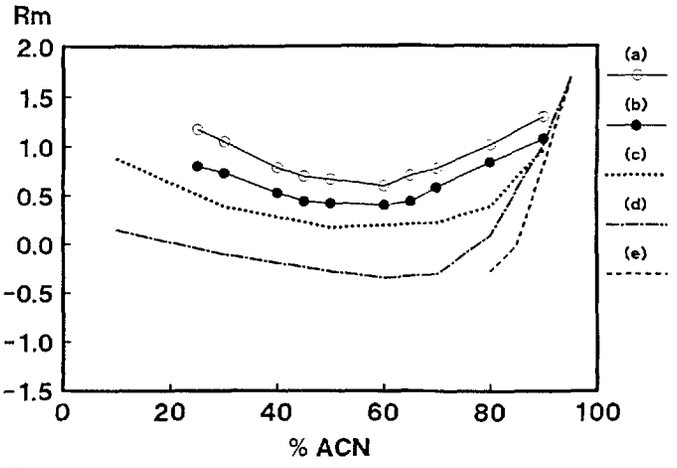

Figure 5

Retention of L/D-Trp sample on various HPTLC plates versus % ACN in H_2O/ACN binary eluent. a) Merck RP-18 $WF_{254}S$ plate covered with copper acetate and LNDH; D-Trp. b) Merck RP-18 $WF_{254}S$ plate covered with copper acetate and LNDH; L-Trp. c) Merck RP-18 $WF_{254}S$ plate covered copper acetate; D/L-Trp. d) Merck RP-18 $WF_{254}S$ plate without modification; D/L-Trp. e) Merck RP-18 end-capped plate; D/L-Trp.

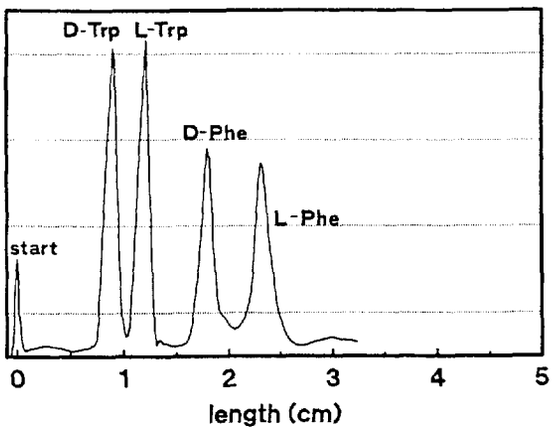

Figure 6

Enantiomer separation of D/L-tryptophan and D/L-phenylalanine on Merck RP-18 $WF_{254}S$ plate covered with copper acetate and LNDH. Eluent: MeOH/ACN/THF/H_2O 7.3:5.9:33.9:52.9. Migration: 5 cm. Sample: 2 µg. Detection: ninhydrin reaction (see Experimental); *in situ* evaluation with TLC/HPTLC Scanner II (Camag), reflectance mode, 550 nm.

Table II. Retention parameters of aromatic amino acid enantiomers on Merck RP-18 $WF_{254}S$ plates covered with copper acetate and LNDH. Solvent: MeOH/ACN/THF/H_2O 7.3:5.9:33.9:52.9.

Amino acid	R_m(L)	R_m(D)	α*
Tryptophan	0.50	0.66	1.33
α-Methyltryptophan	0.56	0.73	1.38
5-Methyltryptophan	0.60	0.75	1.33
6-Methyltryptophan	0.63	0.72	1.19
Phenylalanine	0.07	0.25	1.28
α-Methylphenylalanine	0.18	0.42	1.43
Tyrosine	-0.03	0.13	1.21
α-Methyltyrosine	0.06	0.24	1.27
3-(3,4-Dihydroxyphenyl)alanine (DOPA)	0.08	0.24	1.24

* $\alpha = R_f(L)/R_f(D)$

Conclusions

The separation system described gave good resolution of aromatic, amino-acid enantiomers. It is shown that the newly synthesized, chiral selector, LNDH is suitable for modification of an RP-C_{18} stationary phase and that it has separation powers for racemic amino acid mixtures. Moreover, from a qualitative point of view, the predictions made on the basis of thermodynamic solution data for model systems were in good agreement with the TLC results.

One limitation was that, due to detection problems, it proved impossible to increase the active chiral selector concentration on the plate surface; high spot concentrations were always needed and hence quantitative measurements could not be made. Quantitative studies may be possible on a more efficient separation system, such as a chiral HPLC column containing the chiral selector LNDH. The information from the TLC experiments will make the realization and optimization of more complex HPLC systems easier [37, 38].

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