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3-Amino derivative of β-cyclodextrin: thermodynamics of copper(II) complexes and exploitation of its enantioselectivity in the separation of amino acid racemates by ligand exchange capillary electrophoresis

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Abstract

The systems that the 3-amino derivative of β-cyclodextrin (CD3NH2) forms with the proton, the copper(II) ion and each of the enantiomers of certain amino acids (alanine, phenylalanine, tyrosine and tryptophan) were investigated. The enantioselectivity shown by the potentiometric measurements carried out on the phenylalanine ternary systems was exploited in capillary electrophoresis by ligand exchange capillary electrophoresis (LECE) to obtain the separation of phenylalanine racemate. The tyrosine racemate was also separated by LECE. The comparison between thermodynamic and capillary electrophoresis (CE) results is discussed, in order to get a better insight into the separation mechanism.

Keywords: Enantiomer separation; Copper(II) complexes; β-Cyclodextrins; Amino acids

1. Introduction

Cyclodextrins (CDs) have become an object of interest in a variety of contexts such as in the fields of separation science [1,2], mimicking enzymes [1,3–5] and delivery systems [1,6].

Replacement of the hydroxyl groups of CDs with other functional groups has been shown to improve remarkably the CDs ability to form inclusion complexes, as well as their catalytic properties [1,3–5,7,8]. A large number of functionalized CDs have been described and the improvements so obtained compared to parent CDs have been illustrated [1,3].

Functionalization is also a useful way to improve the CDs ability to form stable metal complexes and in this context different appropriate moieties have been attached to the CD cavity [1,4,8,9]. In the cyclodextrin metal complexes the metal ion can cooperate with the binding properties of CD cavity, as a supplementary recognition site. Copper(II) or nickel(II) complexes of cyclodextrins monofunctionalized with diamines have been used as molecular receptors and enantioselective binding of amino acids has been verified [10–21].

As known, LEC is a technique which was first developed in liquid chromatography [22,23], successively applied to CE [24–26], which is based on the ligand exchange occurring in solution by adding a suitable metal complex. If the ligand of the added complex is chiral, LEC can be used to obtain the separation of racemates, by exploiting

Abbreviations: LEC, ligand exchange chromatography; LECE, ligand exchange capillary electrophoresis; CE, capillary electrophoresis; CD–EKC, cyclodextrin–electrokinetic chromatography; BGE, background electrolyte; Rs, resolution; Sf, selectivity factor; μ, electrophoretic mobility; t_l, migration time of L-enantiomer; t_d, migration time of D-enantiomer; AA, amino acid; CD, cyclodextrin; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; T-ROESY, rotating frame overhauser effect spectroscopy; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate

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the diastereoisomerism between the complexes of the two enantiomers. Moreover, by using different techniques, it was possible to compare spectroscopic evidence and thermodynamic results concerning the formation of the ternary complexes with chromatographic results, thus getting a better insight on the observed enantioselectivity [11,16,17].

Up to now, all the copper(II) complexes we have studied were obtained with 6-derivatised β-cyclodextrins. To go a step further, we turned our attention to the complexing properties of a 3-derivative: here, we investigate on the 3A-amino-3A-deoxy-2A(*S*),3A(*R*)-β-cyclodextrin (CD3NH2) and its copper(II) complex. This compound (where the alternative functionalization was carried out, i.e. at the wider cyclodextrin rim) appears particularly interesting because it can throw light on the opposite influence that, for the 3-derivatised cyclodextrins, the cavity can exert on their coordination ability [27–30]. For the 6-functionalised cyclodextrins it was verified that, mainly for steric reasons, the formation constants of metal complexes are lower than those of the corresponding compounds without the cavity [19,20,31,32], and no involvement of the hydroxyls was observed. Both these two aspects may be quite different in the 3-functionalised cyclodextrins. The steric characteristics of the wider rim are obviously quite different from that of the narrower rim, while the affinity of the secondary hydroxyls, both towards protons and metal ions, may be quite different from that shown by the primary hydroxyls of the narrower rim.

While cyclodextrin intrinsic chirality is often exploited in electrokinetic chromatography (CD–EKC), only recently did we report the first example of chiral separation in capillary electrophoresis by a ligand exchange mechanism (LECE) by using a cyclodextrin derivative as a first coordination ligand [33]. Here, likewise, the enantioselectivity of the copper(II) complex of CD3NH2 was tested in capillary electrophoresis for the separation of amino acid racemate. Furthermore, by a potentiometric study of the corresponding ternary complexes, we can compare the thermodynamics of complex formation with LECE results.

2. Experimental

2.1. Materials

β-Cyclodextrin (Fluka), anhydrous *N*,*N*-dimethylformamide (Aldrich) were used without further purification. Molecular sieves 13X (Aldrich) for the solfonation reactions were activated at 400 °C for 2 h. Thin layer chromatography (TLC) was carried out on silica gel plates (Merck 60-F254). Merck Lichroprep RP-8 (40–60 μm) was used for reversed phase column chromatography. CD derivatives were detected on TLC by UV and by anisaldehyde test. 2-Tosyl-β-cyclodextrin was synthesized as reported in [34], and it was purified by reversed phase chromatography, using a linear gradient H_2O–MeOH as the eluent. The 3A-amino-3A-deoxy-2A(*S*),3A(*R*),-β-cyclodextrin was synthesized by a slightly modified procedure [35].

Synthesis 3A-amino-3A-deoxy-2A(*S*),3A(*R*),-β-cyclodextrin (CD3NH2).

A solution of 200 mg of 2-tosyl β-cyclodextrin [34] and 400 mg of $NaHCO_3$ in 3 ml of water was stirred at room temperature. After 4 h, NH_3 was added (1 ml, 28% solution). The reaction mixture was stirred under nitrogen at 60 °C for 7 h. The solution was evaporated to dryness in vacuo, precipitated in acetone, and purified by elution from a column (35 mm × 900 mm) of CM-Sephadex C-25 column (NH_4^+ form) with water (1 l), then with a 0–0.15 M NH_4HCO_3 linear gradient (total volume 1 l).The appropriate fractions were concentrated to give CD3NH2, Rf = 0.18 (5/2/2/4 PrOH/AcOEt/H_2O/NH_3), yield: 90%. ESI-MS *m*/*e* 1135 ($M + 1$).

^{1}H NMR (D_20, 500 MHz) ppm: 2.89 (dd, 1H, H-3A), 3.45–3.60 (m, 12H, H-2, H-4), 3.68 (m, H, H-2A), 3.73–3.93 (m, 27H, H-3, H-6, H-5), 4.14 (m, 1H, 5A), 4.73 (d, 1H, H-1A, $J_{1,2} = 7.0$ Hz), 4.76 (d, 1H, $J_{1,2} = 4.0$ Hz), 4.92 (d, 1H, $J_{1,2} = 4.0$ Hz), 4.95 (d, 1H, H-1, $J_{1,2} = 3.6$ Hz), 4.96 (d,1H, H-1, $J_{1,2} = 4.0$ Hz), 5.05 (m, 2H, H-1).

2.2. Potentiometric measurements

$Cu(NO_3)_2$ was prepared from $Cu_2(OH)_2CO_3$ by adding a slight excess of HNO_3. Stock solution concentration was determined by EDTA titrations with a murexide indicator. The concentrations of HNO_3 and KOH stock solutions were determined by titration with the primary standard tris(hydroxymethyl)aminomethane (THAM) and potassium hydrogen phthalate, respectively. KNO_3 (Suprapur Merck) was used without further purification. All solutions were prepared with doubly distilled water.

Stability constants for proton and copper(II) complexes were calculated from potentiometric titrations carried out at 25 °C (total volumes: 2.5 cm^3). The KOH solution was added using Hamilton burettes equipped with 0.25 or 0.50 cm^3 syringes. The pH changes were measured using a combined Metrohm 125 microelectrode, standardized on the pH = −log [H^+] scale by titrating HNO_3 solutions with CO_2 free KOH. The ionic strength of all solutions was 0.1 M (KNO_3). The concentrations of the β-cyclodextrin derivative (CD3NH2) and the amino acids ranged from 2.0×10^{-3} to 5.0×10^{-3} M. Duplicate or triplicate titrations were carried out for simple complexes in the 1:1.2 and 1:3.5 Cu/CD3NH2 ratio range and for mixed complexes at a 1:2.7:1 Cu/CD3NH2/AA ratio. Other details were as previously described [36]. Calculations for the electrode system E° values, ligand purity, and HNO_3 excess in the metal stock solution were performed using the Gran method [37] or the least-squares ACBA computer program [38].

All other potentiometric data were handled using the program SUPERQUAD [39], thus minimizing the error-square sum of the differences between measured and calculated

electrode potentials. In this paper, errors in stability constant values are expressed as three times standard deviations. Distribution diagrams were calculated by DISDI program.

2.3. CE measurements

LECE measurements were carried out on a Beckman P/ACE MDQ equipped with a diode array detector. An uncoated fused-silica capillary (Beckman, 50 cm total length, 37.5 cm effective length, 50 μm i.d.) was held at a constant temperature of 20 °C. The system operated at a constant voltage of 12 kV. BGEs for the chiral separation experiments were prepared by dissolving the cyclodextrin derivative ($(0.6–1.8) \times 10^{-3}$ M) in 2.0×10^{-2} M CH_3COONH_4 (pH 6.8) in the presence of $CuSO_4$, Cu^{2+}/CD3NH2 1:1.2 ratio. The sample solution (2.5×10^{-4} M in racemate, 5.0×10^{-5} M in L-enantiomer excess) was obtained by dissolving the analyte in the same condition. Duplicate runs were carried out in the absence of copper(II), namely, by dissolving the cyclodextrin derivative ($(0.6–1.8) \times 10^{-3}$ M) in 2.0×10^{-2} M CH_3COONH_4 (pH 6.8). No separation was attained in these experiments in the absence of copper(II) ion.

The sample was electrokinetically injected (10 kV, for 10 s). Before each experiment, the capillary was flushed (pressure of 2.0×10^6 Pa) with 2.0×10^{-2} M CH_3COONH_4, 0.1 M NaOH and the BGE used in separation. The capillary was rinsed daily with 0.1 M HCl, water, 0.1 M NaOH and finally the used BGE.

Enantiomeric resolution (Rs) and selectivity factor (Sf) were calculated using Eqs. (1) and (2), respectively:

$$\mathrm{Rs} = 2 \times \frac{t_2 - t_1}{w1 + w2} \quad (1)$$

$$\mathrm{Sf} = 2 \times \frac{\mu 2 - \mu 1}{\mu 1 + \mu 2} \quad (2)$$

where t, w and μ are, respectively, migration times, peak widths at the baseline and the electrophoretic mobilities. Resolution values lower than 1 are reported in the table, to permit homogenous comparison with literature data. However, it is our considered opinion that, since a value lower than unity means that a quantitative separation was not attained, showing exactly how much lower than unity the value is, does not appear to be of particular interest. Furthermore, it is not clear how this value should be calculated. In many cases, measurements of the peak width taken in the usual way, by finding the point of intersection between the baseline and the tangent in the peak inflection point, can prove unreliable, this being due to the necessary extrapolation of both the peak and the baseline.

2.4. Spectroscopy

^{1}H NMR spectra were recorded at 25 °C in D_2O with a Varian Inova 500 spectrometer at 499.883 MHz. The ^{1}H NMR spectra were measured by using standard pulse programs from Varian library. In all cases the length of 90° pulse was ca. 7 μs. 2D experiments were acquired using 1 K data points, 256 increments and a relaxation delay of 1.2 s. T-ROESY spectra were obtained using a 300 ms spin-lock time. DSS was used as external standard.

3. Results and discussion

3.1. Characterization of the ligand and of the copper complexes

The CD3NH2 was characterized by NMR (COSY, TOCSY, T-ROESY). The notation system in which the glucose rings are named: A, B, C, D, E, F, and G rings counter clockwise and viewed from the primary hydroxyl side has been adopted.

The ring opening of the manno-epoxide generally gives rise to derivative in position 3 having modified altrose residue. The introduction of one altroside into the cyclodextrin caused unequivalency of the glucose units in the NMR spectrum. After the 3-functionalization in addition to the two groups of peaks observed in the 4.1–3.5 ppm region due to the CD protons, peaks are present at 2.81 and 4.14 ppm due to the H-3A and H-5A (A is the functionalized ring), at 3.68 2A and at 4.73 1A. The coupling constant values between H-1A and H-2A ($J_{1A,2A} = 7.0$ Hz) and between H-2A and H-3A ($J_{2A,3A} = 10$ Hz) indicated that 1A, 2A, 3A protons are axial. Furthermore, the coupling constant value for H-3A with H-4A ($J_{3A,4A} = 3.5$ Hz) indicated an equatorial orientation of the 4A proton. The data compared with the calculated J for D-glucopyranosid rings suggest that the altrose residue is in an $^1C_4 \leftrightarrows {}^0S_2 \leftrightarrows {}^4C_1$ conformational equilibrium shifted towards the $^1C_4$ conformation, as typically described for this class of compounds [40–43]. The involvement of the NH group in the H-bond formation with 2-OH of B ring can be proposed, in the same way for the monoaltrocyclodextrins. The conformation $^1C_4$ where the OH and NH groups are equatorial can be stabilized for the formation of H-bonds with the adjacent rings B and G analogously as in the β-CD.

The protonation constant of CD3NH2 is reported in Table 1, together with the formation constants pertinent to

Table 1
Logarithmic stability constant values for binary systems of CD3NH2 with proton and copper(II) ion at 25 °C and $I = 0.1$ M (KNO_3)

Equilibrium	log β[a]
CD3NH2 + H^+ = [CD3NH2(H)]$^+$	7.631(2)
Cu^{2+} + CD3NH2 = [Cu(CD3NH2)]$^{2+}$	3.50(2)
Cu^{2+} + 2CD3NH2 = [Cu(CD3NH2)$_2$]$^{2+}$	7.50(2)
Cu^{2+} + CD3NH2 = [Cu(CD3NH2)H$_{-1}$]$^+$ + H^+	−2.72(5)
Cu^{2+} + 2CD3NH2 = [Cu(CD3NH2)$_2$H$_{-1}$]$^+$ + H^+	2.00(2)
Cu^{2+} + 2CD3NH2 = [Cu(CD3NH2)$_2$H$_{-2}$] + 2H^+	−4.99(1)

[a] 3σ in parentheses.

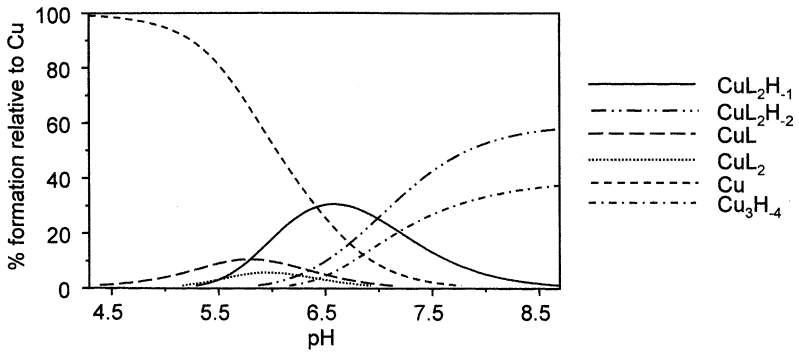

Fig. 1. Distribution diagram for the Cu^{2+}–CD3NH2 system at $[Cu^{2+}] = 5.0 \times 10^{-3}$ M and [CD3NH2] $= 6.0 \times 10^{-3}$ M.

the copper(II)/CD3NH2 binary system. The value found ($\log K = 7.63$) is low for an amino group, but very similar to a simple amino derivative of glucose, the glucosamine. This decrease can be ascribed to hydrogen bond formation in the unprotonated species, that is broken by the nitrogen protonation.

The binary system Cu-CD3NH2 shows the existence of more species between pH 5 and 8.5, and the corresponding values are reported in Table 1. An example of a distribution diagram is reported in Fig. 1, and it is readily evident that the species which are far more abundant are $[Cu(CD3NH2)_2H_{-1}]^+$ and $[Cu(CD3NH2)_2H_{-2}]$, though the simulation was carried out with only a slight excess of ligand (the concentration ratio is 1:1.2). The species deprotonated twice, in particular, is so strong that at the more basic pH values investigated it is the only CD3NH2 species, thus "forcing" the excess of copper(II) to be present as a hydroxylic species. The tendency to give rise to species where the copper(II) is coordinated by two ligand molecules is evidenced by the corresponding values of formation constants. If we compare the value of the unprotonated complex with a ligand molecule (3.50) with that of the corresponding species with two ligand molecules (7.50), we find that this is more than twice the first value. Likewise, comparing the complexes with one and two deprotonated ligands (−2.72 and −4.99, respectively). Statistically, for the species with two ligand molecules, we should have values lower than 7.0 and −5.44, respectively, and this difference shows that there is an extra-stability effect, presumably due to an interaction between the two different molecules of the ligand. This interaction is promoted by the proximity forced by the coordination to the same copper(II) ion. As well, from Fig. 1, the facility of the creation of deprotonated species is clear. The deprotonation, assisted by metal ions, occurs on a hydroxyl near the amino group, and is favored by chelate effect so obtained. This behavior is observed for this class of derivatives [29,30] and for the amino alcohols [44,45].

Formation constants pertinent to the four ternary systems investigated, obtained by adding one of each of the alanine and phenylalanine enantiomers are reported in Table 2. For the systems with each of the alanine enantiomers, only the formation of deprotonated species is observed, and the corresponding formation constants are identical when considering the experimental uncertainty. On the other hand, both the phenylalanine systems show the formation of two different ternary systems. From pH = 5, an unprotonated species starts to be formed, while from pH = 6 the deprotonated species adds, and increasing pH its concentration quickly increases, becoming equal to the other ternary species and being far the most important species at the higher pH values. Formation constants of the ternary complexes of L-phenylalanine have values higher than the corresponding species formed by the D-phenylalanine. In order to underline this difference, typical distribution diagrams, one for each system, are reported in Fig. 2a and b, values of formation degree (α) being referred to the amino acidate, instead of the usual reference to metal ion. It can be seen that a significant higher percentage of $[Cu(CD3NH2)(PheO)]^+$ is formed in the case of L-$PheO^-$ (∼10%) compared to the D-$PheO^-$ species (∼6%).

Table 2
Logarithmic stability constant values of copper(II) ternary complexes of CD3NH2 with L/D-alaninate or phenylalaninate at 25 °C and $I = 0.1$ M (KNO_3)

Equilibrium	$\log \beta$[a]
Cu^{2+} + L-$PheO^-$ + CD3NH2 = $[Cu(CD3NH2)(L\text{-}PheO)]^+$	11.72(4)
Cu^{2+} + D-$PheO^-$ + CD3NH2 = $[Cu(CD3NH2)(D\text{-}PheO)]^+$	11.29(3)
Cu^{2+} + L-$PheO^-$ + CD3NH2 = $[Cu(CD3NH2)(L\text{-}PheO)H_{-1}] + H^+$	5.34(5)
Cu^{2+} + D-$PheO^-$ + CD3NH2 = $[Cu(CD3NH2)(D\text{-}PheO)H_{-1}] + H^+$	4.39(3)
Cu^{2+} + L-$AlaO^-$ + CD3NH2 = $[Cu(CD3NH2)(L\text{-}AlaO)H_{-1}] + H^+$	4.88(5)
Cu^{2+} + D-$AlaO^-$ + CD3NH2 = $[Cu(CD3NH2)(D\text{-}AlaO)H_{-1}] + H^+$	4.85(5)

[a] 3σ in parentheses.

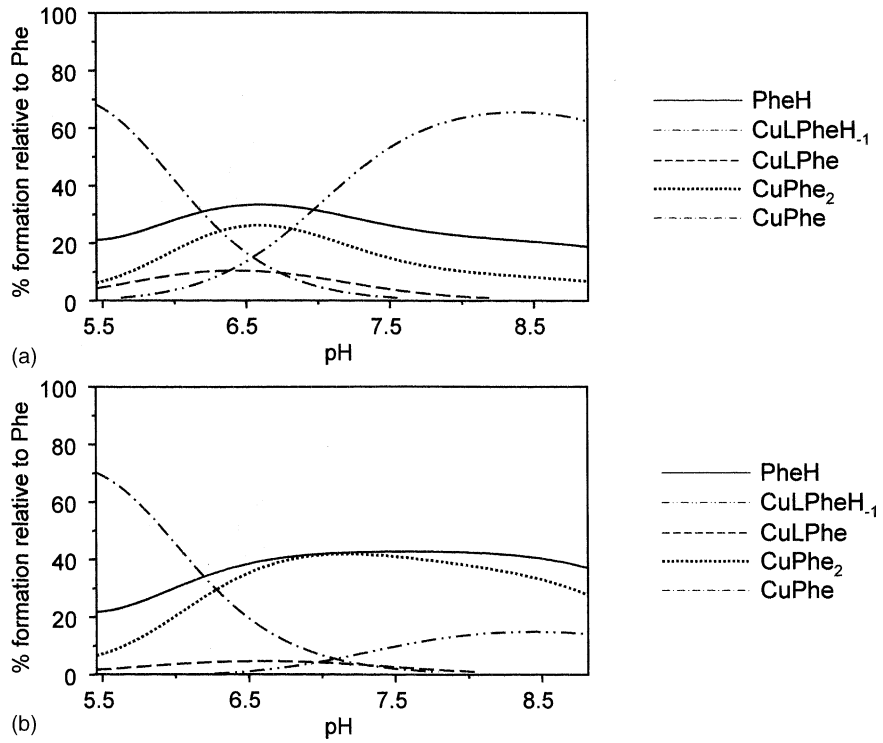

Fig. 2. Distribution diagram for the Cu^{2+}–CD3NH2–AaO^- system: $[Cu^{2+}] = 7.5 \times 10^{-3}$ M, [CD3NH2] = 9.0×10^{-3} M, [Phe] = 1.0×10^{-4} M: (a) L-phenylalanine; (b) D-phenylalanine.

3.2. Enantiomeric separation of amino acids in capillary electrophoresis

In Table 3, we report the results of the LECE experiments, run at different concentrations of the copper(II) complex. Unfortunately, owing to the insufficient absorbance of alanine, it was not possible to study these systems. While at no CD3NH2 concentration was tryptophan racemate separated, for both the other racemates investigated, a discriminating effect can be seen (Figs. 3 and 4). As regards tyrosine, at one only concentration value was it possible to obtain complete separation. As can be seen in the corresponding electropherograms, shown in Fig. 3, the racemate that was better separated is phenylalanine and at the highest concentrations used, complete separation, i.e. resolution value greater than 1, was attained. It can be of interest to observe the very low concentrations necessary to obtain this result.

Table 3
Results of LECE experiments

AA	[CD3NH2] (M)	t_l	t_d	Sf	Rs
Phe	1.8×10^{-3}	10.62	10.78	0.015	1.45
	1.2×10^{-3}	10.40	10.64	0.023	1.71
	8.0×10^{-4}	9.15	9.26	0.012	0.54
	6.0×10^{-4}	8.44	8.48	0	0
Tyr	1.8×10^{-3}	10.53	10.69	0.015	1.33
	1.2×10^{-3}	9.97	10.06	0.009	0.47
	8.0×10^{-4}	8.84	8.90	0	0
	6.0×10^{-4}	8.07	8.07	0	0
Trp	1.8×10^{-3}	9.88	9.88	0	0
	1.2×10^{-3}	9.38	9.38	0	0
	8.0×10^{-4}	8.55	8.55	0	0
	6.0×10^{-4}	8.01	8.01	0	0

For the definition of Sf and Rs, see Section 2.

In order to understand the detailed mechanism of ligand exchange that gives rise to racemate separation, we will analyze the equilibria occurring in solution during the electrophoretic runs. As can be seen in the distribution diagram reported in Fig. 2, at the pH value of the BGE used for these electrophoretic runs (pH = 6.8), significant concentrations of two different complex species are present. Thus, the first thing to understand concerns the specific complex involved in separation. If we consider that the free ligand at that pH value is neutral and that the deprotonated complex is neutral too, it is quite obvious that this complex cannot give rise to any separation, and that, on the contrary, its formation should not even modify the migration time of the analytes. Thus, it is only the unprotonated complex, which is monocationic, that, by the usual difference in the weighted mean migration time between the two enantiomers can yield the racemate separation. The result of electrophoretic separations, which show that the L-enantiomer has a migration time shorter than the D-enantiomer is in full agreement with the stability constants found: the higher value for the L-enantiomer complex suggests that a higher ligand fraction is coordinated, giving rise to a higher average cationic charge and thus to a shorter migration time.

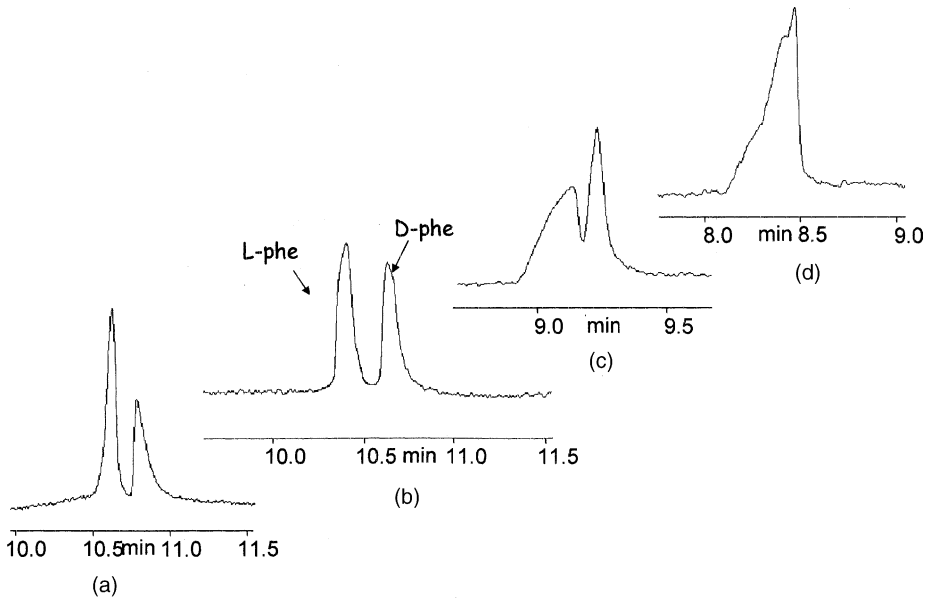

Fig. 3. Electropherograms ($\lambda = 200$ nm) of phenylalanine racemate (excess of L-Phe) in the presence of copper(II) and CD3NH2 at pH 6.8: [CD3NH2] is (a) 1.8×10^{-3} M; (b) 1.2×10^{-3} M; (c) 8.0×10^{-4} M; (d) 6.0×10^{-4} M.

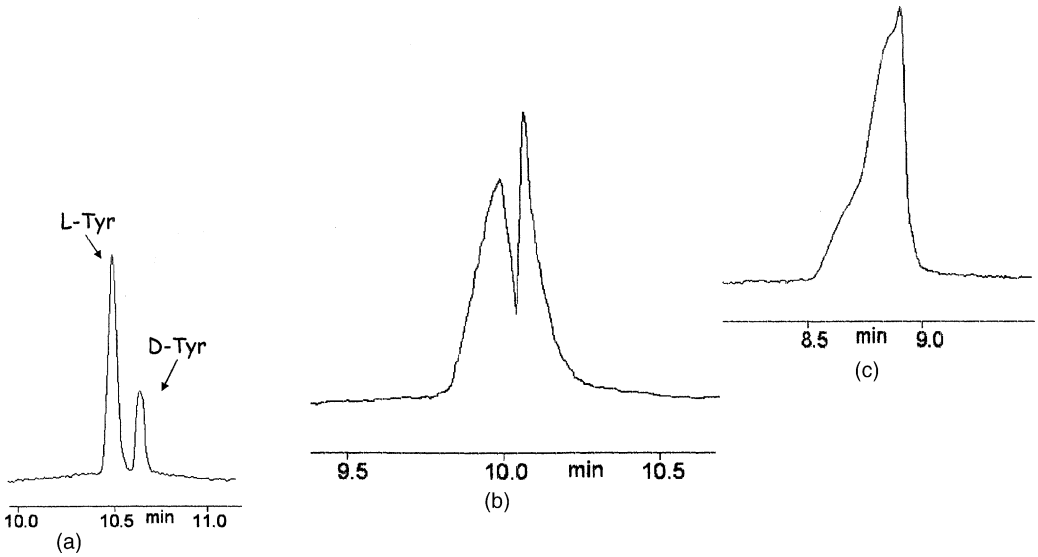

Fig. 4. Electropherograms ($\lambda = 220$ nm) of tyrosine racemate (excess of L-Tyr) in the presence of copper(II) and CD3NH2 at pH 6.8: [CD3NH2] is (a) 1.8×10^{-3} M; (b) 1.2×10^{-3} M; (c) 8.0×10^{-4} M.

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