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Chiral separation based on ligand-exchange capillary electrophoresis using a copper(II)-L-ornithine ternary complex as selector

A ligand-exchange capillary electrophoresis was explored, with L-ornithine as the ligand and copper(II) as the central ion. Its applicability was demonstrated with underivatized and dansyl amino acids, a dipeptide, and drugs with amino alcohol structure. The enantioselectivity was found to be strongly dependent on pH and copper(II)-L-Orn complex concentration. Due to the adsorption of the positively charged species onto the capillary inner walls, the chiral separation selectivity is very high while the efficiency is relatively low. Permanent 1,3-propanediamine-coated capillaries show an improved separation efficiency and theoretical plate numbers increasing from 10^4 to 10^5. Similar phenomena were observed when sodium dodecyl sulfate (SDS) micelles were added to the copper(II) complex solution. The poor separation efficiency of chiral compounds in uncoated capillaries may result from the low rate of the ligand-exchange reactions, and the high enantioselectivity may derive from the complexing process in the adsorbed phase.

Keywords: Chiral separation / Ligand-exchange capillary electrophoresis / Ornithine
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1 Introduction

Chiral ligand-exchange chromatography (LEC), introduced by Davankov and Rogozhin [1] in the early 1970s, was the first high-performance liquid chromatography (HPLC) technique which allowed the baseline resolution of enantiomers having two polar groups that are able to form chelate complex with transition metal cations, such as amino acids. However, the efficiency of the ligand-exchange (LE) HPLC columns was relatively low resulting from the slow kinetics of LE although its enantioselectivity was generally excellent [2]. High separation efficiency, an inherent characteristic of capillary electrophoresis (CE), can be very advantageous in this case. The first application of this principle in CE was reported by Gassmann *et al.* [3, 4] using histidine- or aspartame-copper(II) complexes as chiral selectors for a successful resolution of dansyl amino acids. In 1994, Desiderio [5] extended the application of chiral LE-CE to hydroxy acids using L-hydroxyproline (Hypro) and aspartame Cu(II) complexes as chiral selectors. So far, a variety of chiral copper(II) complexes have been successfully used as chiral resolving agents for enantiomeric separation of amino acids [6], amino alcohols [7], organic acids [8], diamines and small peptides [9], such as tataric acid [8], L-Arg [10], and L-proline and its derivatives [6, 11].

Micellar electrokinetic chromatography (MEKC), introduced by Terabe [12] in 1984, is a useful technique for the CE analysis of uncharged compounds. Chen and Lin [13–16] published on the simultaneous separation of positional and optical isomers of fluoro-DL-phenylalanine, tyrosine as well as tryptophan derivatives, and a mixture of underivated and dansylated amino acids using LE-MEKC. Interestingly, the addition of the achiral surfactant SDS not only significantly improved the resolution but led to the reversal of the enantiomer migration order (EMO) for phenylalanine and tryptophan enantiomers. Similar observations regarding reversal of EMO were reported by Lu and Chen [17] in the course of the separation of underivatized amino acids using copper(II)-L-Lys as a selector with and without SDS addition. As a hybrid mode possessing both the advantage of high enantioselectivity of the LE mechanism and the main advantages of MEKC, LE-MEKC allows the manipulation of the selectivity for large classes of neutral and charged compounds, making separations possible that otherwise are not feasible by using only the mode of LE or MEKC.

In recent years, a few reports dealing with enantioselective LE with capillary electrochromatography (LE-CEC) have been published using chiral stationary phases (CSPs), *i.e.*, polymer-based continuous beds [18] or

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Abbreviations: Dns-AA, dansyl amino acid; **EMO**, enantiomer migration order; **LE-CE**, ligand-exchange capillary electrophoresis; **L-Orn**, L-ornithine; **PDA**, propanediamine

monolithic columns [19]. There is no doubt that LE-CEC mode is a promising technique and offers attractive possibilities for chiral separation. However, this technique suffers from the tedious procedure and a number of problems still need to be resolved compared to relatively mature techniques like LE-CE and LE-MEKC.

LE-CE has been used in chiral separation for almost 20 years since it was firstly reported in 1985 [3], but until now, the compounds used as ligands for LE-CE are very few, resulting in the slow development of LE-CE. In the present work, we describe the use of a novel enantiomeric ternary complex of copper(II)-L-ornithine as chiral selector for the chiral separation of underivatized and dansyl amino acids, a dipeptide, and amino alcohol enantiomers.

2 Materials and methods

2.1 Apparatus

Enantioseparation was carried out on a Beckman P/ACE MDQ capillary electrophoresis system (Beckman, Fullerton, CA, USA) equipped with a photodiode array detector. Sample detection was performed at 214 nm. Beckman 32 Karat software (Fullerton, CA, USA) was used for data collection and processing. Uncoated fused-silica capillaries, 50 μm ID, 375 μm OD and 57 cm in length, from Yongnian Optical Fiber Work (Hebei, China) were used throughout the experiments. A detection window was created at 50 cm from the capillary inlet by removing the polyimide coating. The capillary temperature was maintained at 25°C by the cooling system of the CE instrument. The hydrostatic injection mode (5 s, 0.5 psi) was used for the injection of the standard sample. Applied voltage was 25 kV. Dimethyl sulfoxide (DMSO) was used to mark the electroosmotic flow (EOF). Samples were injected hydrodynamically at 0.5 psi for 5 s and separated at 25 kV. To clean and activate the inner surface, new capillaries were flushed successively for 5 min with methanol, 5 min with 0.1 mol·L^{-1} HCl, 10 min with 0.1 mol·L^{-1} NaOH, and 5 min with H_2O. Daily before use, the capillary was rinsed for 2 min with 0.1 mol·L^{-1} NaOH, 2 min with H_2O, and 4 min with running electrolyte. Between analyses, the capillaries were rinsed with running electrolyte at 20 psi for 2 min.

2.2 Chemicals

L-Ornithine (Orn) was of biochemical-reagent grade from the Institute of Shanghai Chemical Reagents Factory (Shanghai, China). Copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was from Kanto Chemical (Tokyo, Japan). Dansyl DL-amino acids (Dns-DL-AAs), including aspartic acid (Asp), glutamic acid (Glu), leucine (Leu), methionine (Met), α-amino-*n*-butyric acid (Nbu), norleucine (Nleu), norvaline (Nval), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), and valine (Val), were purchased from Sigma (St. Louis, MO, USA). A dipeptide, DL-alanine-DL-phenylalanine (Ala-Phe) and underivatized DL-Phe were from Tokyo Kasei Kogyo (Tokyo, Japan); DL-Trp was from Nacalai Tesque (Kyoto, Japan). The enantiomers of pseudo-ephedrine and ephedrine were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SDS was obtained from Acros Organics (Pittsburgh, PA, USA). Other chemicals were all of analytical-reagent grade obtained from the Beijing Chemical Factory (China) and used as received. Standard solutions of amino acids were obtained by dissolving amino acids in 20 mmol·L^{-1} ammonium acetate (NH_4OAc) solution and the concentrations of all stock solutions were 1.0 mmol·L^{-1}. Sample solutions were prepared by dilution of stock solutions in 20 mmol·L^{-1} NH_4OAc at a concentration of 10^{-4} mol·L^{-1}. The running electrolyte used for the enantiomeric separation, unless otherwise noted, consisted of 1 mmol·L^{-1} $CuSO_4$, 2 mmol·L^{-1} L-Orn without or with 30 mmol·L^{-1} SDS in 20 mmol·L^{-1} NH_4OAc adjusted to a desired pH by addition of 1 mol·L^{-1} aqueous ammonia or 1 mol·L^{-1} acetic acid. Water used to prepare sample and buffer solutions was freshly deionized by a EASYpure LF water purification system with a 0.2 μm fiber filter (Barnstead, Dubuque, IA, USA).

2.3 Calculation

Electroosmotic mobility, μ_{eo}, was determined by the measurement of the migration time of 5% DMSO solution, t_{eo}, according to the following expression:

$$\mu_{eo} = (L_t \cdot L_d) / (V \cdot t_{eo}) \quad (1)$$

where L_t is the total capillary length, L_d is the capillary length from the injection inlet to the detector, and V is the applied voltage. Resolution of each Dns-AA enantiomer was calculated by measuring the *Rs* value, which is defined by the following equation:

$$Rs = (t_2 - t_1)/(w_{1/2,1} + w_{1/2,2}) \quad (2)$$

where t_1 and t_2 are the migration times, and $w_{1/2,1}$ and $w_{1/2,2}$ are the peak widths at the half-height of the enantiomers, respectively.

3 Results and discussion

3.1 Chiral separation using capillary zone electrophoresis (CZE)

Orn, having a carboxyl and two amino groups, is a basic amino acid with an isoelectric point (p*I*) at 9.7. As a ligand, L-Orn can interact with Cu^{2+} *via* its *O*- and *N*-atom ligand in complexation reactions. When such a copper(II) complex was used as chiral additive to the running electrolyte for CZE, the enantiomers of amino acids could easily be resolved. Figure 1a shows that the separation selectivity is very high while the efficiency is relatively low (N = 2.5×10^4–6×10^4). Below pH 8.0, Orn is present as the positively charged form of LH_2 [20]. Lower separation efficiency is porbably due to the adsorption of the positively charged species onto the capillary inner walls. A nonuniform ξ potential over the length of the capillary due to adsorption may cause additional band-broadening by local variation of the EOF. To avoid these negative effects, the surface in the fused-silica capillaries must be deactivated for suppression of this undesirable wall interaction of charged molecules. A permanant 1,3-propanediamine (PDA) coating of the capillary inner wall was generated according to the method previously reported [21]. Figure 1b gives the results of the separation of Dns-AAs in the coated capillaries. A PDA coating gives an improved efficiency of the five Dns-AAs. The amino acids now migrate with theoretical plate numbers of 4.6×10^5–5×10^5. However, suppression of peak-broadening did not result in high-resolution separation of these compounds, but on the contrary, in a decrease of separation selectivity. The mechanism of LE is based on the formation of diastereomeric ternary mixed metal complexes between the chiral selector ligand and the analytes. In uncoated capillaries, LE reactions proceed in free solution and at capillary inner wall simultaneously.

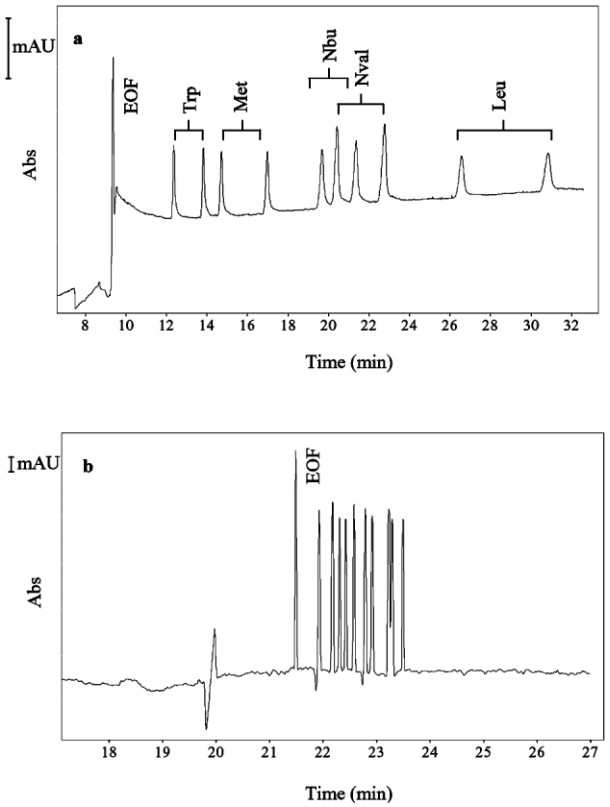

Figure 1. Electropherograms of some Dns-AAs. (a) Uncoated fused-silica capillaries and (b) permanent PDA-coated capillaries. Conditions: 1 mmol·L^{-1} $CuSO_4$, 2 mmol·L^{-1} L-Orn, 20 mmol·L^{-1} NH_4OAc at pH 8.0; injection, 0.5 psi × 10 s; separation voltage, 25 kV (438 V/cm); capillary, 57 cm × 50 μm ID; capillary temperature, 25°C.

Free solution: $A^s + ML^s \rightleftharpoons AML^s$

Capillary wall: $A^w + ML^w \rightleftharpoons AML^w$

Here, L is the selector (L-ornithine), M is the metal ion (Cu^{2+}), and A is the analyte. LE in the bulk solution obviously takes place at higher rates compared with the situation where the chiral ligand is adsorbed onto the capillary wall, while in PDA-coated capillaries LE reactions proceed only in free solution. The poor efficiency of the separation in uncoated capillaries may result from the low rate of the LE reactions. Our results suggested that the enantioselectivity of complexation reactions in the adsorbed phase was higher than those in the free bulk solution. These results are similar to the mechanism reported for LE-CE using complexes as chiral additives or chiral stationary phases [2]. The separation in coated capillaries is much more inconvenient to carry out than in uncoated capillaries. Therefore, the following experiments were performed using uncoated fused-silica capillaries for optimizing some critical conditions.

3.2 Effect of pH on the separation

The effect of the pH value of the running electrolyte was investigated using Dns-Trp and Dns-Met as model analytes. The dependence of resolution of DL-AAs on the pH value of the running electrolyte was studied over the range of 4.0–11.0 using 20 mmol·L^{-1} NH_4OAc buffer consisting of 1 mmol·L^{-1} Cu^{2+} and 2 mmol·L^{-1} L-Orn. As shown in Fig. 2, the separation of Dns-AAs increased apparently with increasing the pH range from 4.0 to 8.0, then it decreased at pH values $>$ 8.0. The distinct maximum of the chiral separation could be achieved at pH 8.0 for all analytes. When the pH increases from 4.0 to 8.0, the stability of the formed Cu(II)-L-Orn complex will be increased. Thus, the chiral resolution should be enhanced.

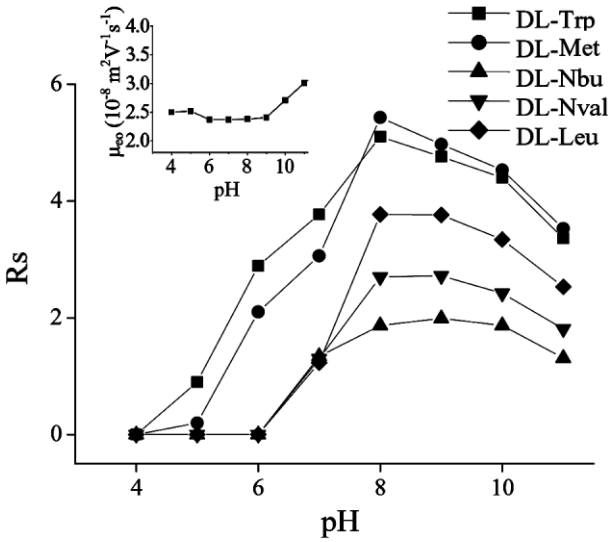

Figure 2. Influence of pH on the EOF (the inserted) and resolution of Dns-AAs. Buffer: 20 mmol·L^{-1} NH_4OAc containing 1 mmol·L^{-1} $CuSO_4$, 2 mmol·L^{-1} L-Orn at different pH values.

But further increasing the pH will lead to too high stability of the Cu(II)-ligand complex, which makes it difficult for the analyte enantiomers to replace ligands in the Cu(II)-ligand complex. These effects result in a maximum pH of 8.0 for the chiral separation. In addition, the pH dependence of the EOF was measured using DMSO as a marker. As shown in the inserted Figure of Fig. 2, at pH 4.0–10.0, the EOF was nearly constant at a value of 2.4×10^{-8} $m^2V^{-1}s^{-1}$. This might be due mainly to the adsorption of positively charged amino groups of Orn to the capillary inner wall when pH $<$ 10 (around the p*I* of Orn, 9.7). Beyond pH 10, the amino propyl group of Orn deprotonated and EOF increases markedly. As a result, the migration time of Dns-AAs was almost unaffected in the pH range of 4.0–10.0 and increased markedly at higher pH values resulting from the increase of electroosmotic mobility. Taking resolution and migration time simultaneously into consideration, a running buffer at pH 8.0 was selected to yield better resolution and shorter separation times.

3.3 Concentration of Cu(II)-L-Orn complex

To determine the optimum selector concentration, the effect of Cu(II)-L-Orn complex concentration was studied in the range from 1 mmol·L^{-1} to 10 mmol·L^{-1} at increments of 2 mmol·L^{-1} while the molar ratio of copper to the chelating amino acid was kept always at 1:2. The results reported in Fig. 3 show that the migration time of Dns-AAs increased with increasing the concentration of

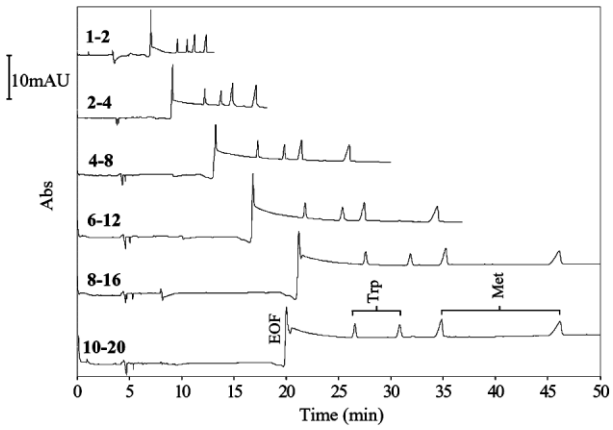

Figure 3. Influence of concentration of Cu(II)-L-Orn complex on EOF and separation of Dns-Trp and Dns-Met. Buffer, 20 mmol·L^{-1} NH_4OAc, pH 8.0; concentration ratio of Cu^{2+} to L-Orn, 1:2–10:20.

Cu(II) complex in the running electrolyte. This is because higher Cu(II)-L-Orn complex concentrations increase the adsorption of cations in the buffer onto the capillary wall and decrease the double-layer thickness. Therefore, the EOF decreased as a result of decreased zeta potential and higher buffer conductivity, which in turn prolonged the migration time. The resolution between each pair of AAs also improved steadily. This demonstrates that an increase of chiral selectors enables the formation of the ternary complex and increases selectivity. In our experiment, 1 mmol·L^{-1} selector concentration was favorable to allow a good resolution for the investigated AAs.

3.4 Application of CZE

The developed method was applied to the separation of a number of enantiomers using the conditions optimized in Section 2; the results are shown in Table 1 and Fig. 4, respectively. As can be seen from Table 1, two underivatized amino acids and 11 pairs of different dansyl amino acids could be successfully resolved. The migration velocity of underivatized amino acids is faster than that of the Dns-AAs. AAs are all negatively charged $<$ pH 8.0 and show a negative mobility. Upon complexing with selectors, the formed mixed complexes are positively charged and will move faster than the free AAs. Thus, the underivatized AAs form more stable ternary complexes with Cu(II)-L-Orn than the Dns-AAs.

In addition to AAs, a dipeptide was also resolved. Figure 4a shows a baseline separation obtained with DL-Ala-DL-Phe. The enantiomers of ephedrine and pseudoephedrine are central nervous stimulants with amino alcohol structures. The chiral separation of these drugs is

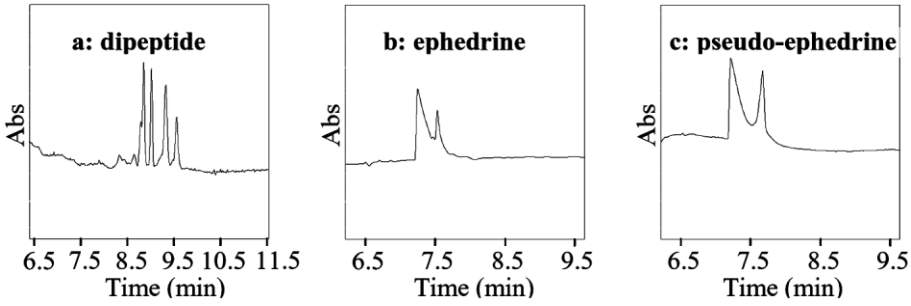

Figure 4. Electropherograms of (a) Ala-Phe, (b) ephedrine, and (c) pseudo-ephedrine enantiomers. Running electrolyte, 20 mmol·L^{-1} NH_4OAc, 1 mmol·L^{-1} Cu^{2+}, and 2 mmol·L^{-1} L-Orn, pH adjusted to 8.0.

Table 1. Separation data for dansyl amino acids. Migration times of the eluted enantiomers ($t_1$and t_2), separation factor (α) and resolution (Rs) are given

Analyte	t_1(min)	t_2(min)	$\alpha(t_2/t_1)$	*R*s
Trp	4.754	5.458	1.15	1.4
Phe	5.888	6.112	1.04	1.1
Dns-Phe	11.192	11.854	1.06	1.8
Dns-Trp	12.179	13.646	1.12	5.1
Dns-Thr	12.867	13.004	1.01	0.8
Dns-Asp	14.367	14.479	1.08	0.6
Dns-Met	14.538	16.804	1.16	5.4
Dns-Nbu	19.504	21.183	1.09	1.9
Dns-Nval	20.250	22.592	1.12	6.0
Dns-Nleu	20.625	21.764	1.06	2.5
Dns-Leu	26.387	30.663	1.16	2.7
Dns-Val	28.096	29.235	1.04	3.8
Dns-Glu	30.333	31.475	1.04	2.1

Migration time of DMSO was 8.554 min.

of great interest since the enantiomers exhibit quantitative and qualitative differences in pharmacological activity. D-(+)-ephedrine, for example, is much more potent in its stimulating action than L-(−)-ephedrine[22]. The resolution of ephedrine and pseudo-ephedrine enantiomers with the running electrolyte supplemented with the Cu(II) complex are demonstrated in Figs. 4b and c, respectively.

3.5 MEKC

CZE is easier to carry out than MEKC, but its separation efficiency was poor because of the adsorption of the positively charged side chain of Orn onto the capillary wall. This negative effect could be prevented when SDS was added to the electrolyte solution. In MEKC, the negatively charged SDS micelles interact with positively charged amino groups through electrostatic interaction, which suppresses the adsorption and in turn may enhance the separation efficiency. 30 mmol·L^{-1} SDS was added as surfactant to the Cu(II) electrolyte solution (Fig. 5). The separation of seven Dns-AAs by MEKC without Cu(II)-L-Orn complex was performed but the resolution and peak shape were poor (Fig. 5a). When the Cu(II)-L-Orn complex was present in the running electrolyte, *i.e.*, the LE-MEKC mode was performed, this poor separation was greatly improved with prolonged migration times (Fig. 5b). Clearly, the separation efficiency greatly increased and the plate numbers of the tested analytes were much higher than that by CZE. However, the separation selectivity by LE-MEKC decreased significantly and the Dns-AAs tested could not be completely resolved.

When introducing SDS micelles into the electrolyte, the Cu^{2+} complexes will partition between the aqueous phase and the micellar phase and chiral recognition is based on the synergic influences of differences in the complex formation constant and the partition coefficient of different enantiomers. In LE-MEKC, the elution order of enantiomers is basically dependent on both the stability of the ternary complexes and the partition coefficient of the enantiomers associated with the chiral selector of

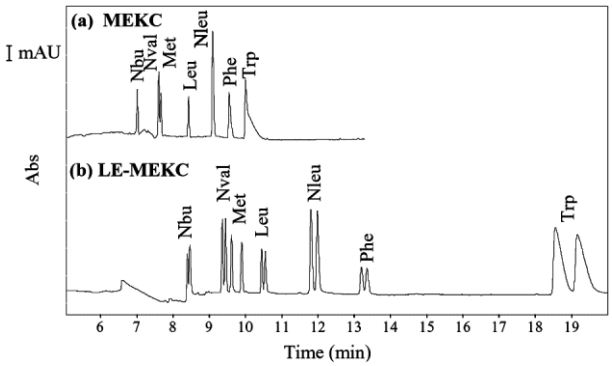

Figure 5. Electropherograms of separation of seven pair Dns-AAs enantiomers by (a) MEKC without or (b) with Cu(II)-L-Orn complex. Conditions: (a) buffer, 20 mmol·L^{-1} NH_4OAc, and 30 mmol·L^{-1} SDS, at pH 8.0; (b) buffer, 20 mmol·L^{-1} NH_4OAc, 1 mmol·L^{-1} $CuSO_4$, 2 mmol·L^{-1} L-Orn, and 30 mmol·L^{-1} SDS at pH 8.0.

Cu(II) complexes between the SDS micellar phase and the bulk electrolyte phase. The migration order is thought to be mainly dependent on the partition coefficient [23]. The compound with the most hydrophobic side chain is partitioned between the micelle and the buffer with the greatest distribution percentage in the micelle and will show the highest retention in the micellar phase. Our results showed that the migration order of AAs in the present system is dependent on the hydrophobicity of the analyte and the elution order of these AA derivatives simply agrees with the hydrophobic nature of the side chains. In addition to a reversal of the migration order of the analytes according to their hydrophobicity, a reversal of the EMO was observed. Figure 6a displays an electropherogram of Dns-Phe obtained by using L-Orn as the ligand of the Cu(II) complex without SDS; the L-enantiomer migrates faster than the D-enantiomer. When 30 mmol·L^{-1} SDS was present in the running solution, the D-form becomes faster than the L-form (see Fig. 6b). In addition, the optical resolution by LE-CE was better than that by LE-MEKC. These facts are probably due to the hydrophobic and electrostatic interaction of Dns-AAs with the negatively charged hydrophobic SDS micelles.

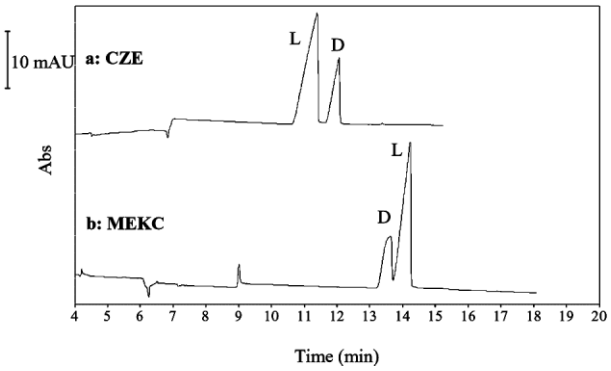

Figure 6. Elution order of Dns-DL-Phe (a) with and (b) without SDS in the buffer. Ratio of Dns-L-Phe to Dns-D-Phe in the sample, 2:1. Conditions: (a) buffer, 1 mmol·L^{-1} $CuSO_4$, 2 mmol·L^{-1} L-Orn, 20 mmol·L^{-1} NH_4OAc at pH 8.0; (b) buffer, 1 mmol·L^{-1} $CuSO_4$, 2 mmol·L^{-1} L-Orn, 20 mmol·L^{-1} NH_4OAc, and 30 mmol·L^{-1} SDS at pH 8.0.

4 Concluding remarks

An optical resolution system for enantioseparation using a Cu(II)-L-Orn complex as chiral selector has been developed. The chiral complex shows effective enantioresolution of Dns-AAs, underivatized AAs, a dipeptide of Ala-Phe, and ephedrine and pseudo-ephedrine enantiomers. This work will inspire to develop a more useful CE chiral separation system and further efforts will be made to extend the applications of this method to real samples.

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