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Chiral ligand exchange high-speed countercurrent chromatography: mechanism, application and comparison with conventional liquid chromatography in enantioseparation of aromatic α -hydroxyl acids

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Abstract

This work concentrates on the separation mechanism and application of chiral ligand exchange high-speed countercurrent chromatography (HSCCC) in enantioseparations, and comparison with traditional chiral ligand exchange high performance liquid chromatography (HPLC). The enantioseparation of ten aromatic α -hydroxyl acids were performed by these two chromatographic methods. Results showed that five of the racemates were successfully enantioseparated by HSCCC while only three of the racemates could be enantioseparated by HPLC using a suitable chiral ligand mobile phase additive. For HSCCC, the two-phase solvent system was composed of butanol-water (1:1, v/v), to which *N*-*n*-dodecyl-L-proline was added in the organic phase as chiral ligand and cupric acetate was added in the aqueous phase as a transition metal ion. Various operation parameters in HSCCC were optimized by enantioselective liquid-liquid extraction. Based on the results of the present studies the separation mechanism for HSCCC was proposed. For HPLC, the optimized mobile phase composed of aqueous solution containing 6 mmol L⁻¹ L-phenylalanine and 3 mmol L⁻¹ cupric sulfate and methanol was used for enantioseparation. Among three ligands tested on a conventional reverse stationary phase column, only one was found to be effective. In the present studies HSCCC presented unique advantages due to its high versatility of two-phase solvent systems and it could be used as an alternative method for enantioseparations.

Keywords

Countercurrent chromatography; Chiral ligand exchange; Enantioseparation; Aromatic α -hydroxyl acids; Separation mechanism

1. Introduction

Development of an efficient method for enantioseparation of pure enantiomeric substances is of increasing interest in the field of pharmaceutical research, especially in the initial stage of seeking for a new drug candidate. Among the various chromatographic techniques for the separation of racemate, chiral ligand exchange liquid chromatography is one of the most

stereoselective separation methods. It was first reported by Hare and Gil-Av, for resolution of underivatized α -amino acids [1], where racemic α -amino acids were separated with the addition of a copper (II) proline complex to the eluent using a cation-exchange column. The chiral ligand was chemically bonded with stationary phase for separation of optical isomers of underivatized amino acids [2, 3]. The chiral separation proceeds via intermediate diastereomeric complex formation with metal ions, to which one of the enantiomers forms a coordinative bond. Because of this requirement, chiral ligand exchange chromatography was mainly used for enantioseparation of enantiomers of α -amino acids and α -hydroxy carboxylic acids [4, 5]. Generally, chiral ligand exchange liquid chromatography could be accomplished with two alternative ways. The first choice is to use a chiral ligand column, in which the packing had been chemically bonded with chiral ligand, and the mobile phase containing transition metal ion. The second one is to use a common achiral column, such as reverse phase packing, and the mobile phase containing the chiral ligand with a metal ion (chiral ion pair). The first method usually provides better chromatographic stability and repeatability, but its cost was higher than the second method and utility was extremely limited because of its specific chiral ligand in the column. For the initial stage of new drug research, the second method has been more widely used because of its lower cost and higher diversity.

With the increasing demand of pure enantiomeric substance, preparative chiral chromatographic techniques become increasingly urgent. Since the preparative HPLC generally requires high capital investment, the use of an alternative preparative chromatography with lower cost is preferred. Countercurrent chromatography belongs to liquid-liquid partition chromatography with the unique advantages of being easy to be scaled up with lower cost. But modern countercurrent chromatography techniques, such as high-speed countercurrent chromatography (HSCCC), and high performance countercurrent chromatography (HPLCCC), have not become the popular chromatographic method for chiral separations because of HSCCC's inherent lower theoretical plates of the separation column [6–8]. Generally, it needs separation factor greater than 1.4 to achieve successful enantioseparation. Unfortunately, enantioseparation factor is always less than 1.4 for chiral chromatographic separations. The value of enantioseparation factor for enantiomers is mainly dependent on the chiral selector. High enantioselectivity occurs only when good chiral selector along with suitable biphasic solvent system is available for enantiomers. Chiral selectors used in CCC generally come from other techniques, such as liquid chromatography, capillary electrophoresis, and enantioselective liquid-liquid extraction. Most of the chiral selectors being successfully used for enantioseparations by these techniques were found to be ineffective in enantioseparation by CCC, and only those chiral selectors with high chiral recognition could be successfully used in enantioseparation by CCC. However, the large loading capacity and diverse usage of countercurrent chromatography greatly outshine other various kinds of chromatographic techniques once the method was successfully applied in enantioseparations.

Formation of diastereomeric coordination ternary complex among enantiomer, transitional metal or nonmetallic ion and chiral ligand is one of the most powerful chiral recognition methods because coordination bonds generally shows much higher stereoselective binding force than other inter-molecular forces, such as hydrogen bonds, van der Waals' force and

dipole-dipole interactions. Only two literatures could be available about application of traditional CCC in enantioseparation based on the chiral ligand exchange. Two racemic α -amino acids, including DL-isoleucine and DL-valine, were reported to be successfully enantioseparated by droplet CCC and countercurrent solvent extraction with the chiral ligand exchange method [9, 10]. Meanwhile, the two racemic amino acids could also be well enantioseparated by conventional chiral ligand exchange liquid chromatography, but, no separation mechanism about chiral ligand exchange CCC was reported in those literatures. Droplet CCC and countercurrent solvent extraction yield much lower separation efficiency than modern HSCCC. In this manuscript, we would like to apply HSCCC in enantioseparation of ten racemic aromatic α -hydroxyl acids using the chiral ligand exchange method, for which the separation mechanism was proposed in this paper. . Meanwhile, enantioseparations of the ten racemates by chiral ligand exchange HPLC with the conventional reverse stationary phase were also investigated. Interestingly, it was found that some of the racemic aromatic α -hydroxyl acids which could not be enantioseparated by HPLC with a chiral ligand or a transitional metal ion as a chiral mobile phase additives, could be well enantioseparated by chiral ligand exchange HSCCC. The chemical structures of the ten racemic aromatic α -hydroxyl acids and the chiral ligand used in the present research were shown in Fig. 1.

2. Experimental Section

2.1. Apparatus

The TBE-20A HSCCC apparatus was used (Shanghai Tauto Biotechnology, Shanghai, China). It was equipped with a set of three analytical multilayer coils connected in series with a total column volume of 20 mL. The other parameters for the apparatus had been described earlier [11]. The apparatus was installed within a vessel that maintains column temperature by a Model SDC-6 constant-temperature controller (Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China). The solvents were pumped into the column with a model s-1007 constant-flow pump (Beijing Shengyitong Technique, Beijing, China). Continuous monitoring of the effluent was achieved with a model UVD-200 detector (Shanghai Jinda Biotechnology Co., Ltd., Shanghai, China), and SEPU3010 workstation (Hangzhou Puhui Technology, Hangzhou, China) was employed to record the chromatogram.

The HPLC apparatus used was a CLASS-VP Ver.6.1 system (Shimadzu Corporation, Kyoto, Japan) comprised of a Shimadzu SPD10Avp UV detector, a Shimadzu LC-10ATvp Multisolvant Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, and a CLASS-VP Ver.6.1 workstation.

The pH value was determined with a portable Delta 320-s pH meter (Mettler-Toledo, Greifensee, Switzerland).

2.2. Reagents

Mandelic acid, 4-bromomandelic acid, 4-methoxymandelic acid, α -cyclohexylmandelic acid, α -cyclopentylmandelic acid, 4-hydroxymandelic acid, L-proline, L-phenylalanine, L-alanine and sodium cyanoborohydride were purchased from J&K Scientific Ltd. (Shanghai,

China). 3-Chloromandelic acid, 4-hydroxy-3-methoxymandelic acid, 2-chloromandelic acid and α -methylmandelic acid were purchased from Tokyo Chemical Industry Co, Ltd. (Tokyo, Japan). Hydroxypropyl- β -cyclodextrin and sulfonyl butyl ether- β -cyclodextrin (degree of substitution 7.5 and 6.9, respectively) were purchased from Qianhui Fine Chemical & Co. Inc. (Shandong, China). All organic solvents used for HSCCC separation were of analytical grade. Acetonitrile and methanol used for HPLC analysis were of chromatographic grade. Water used for HPLC study was redistilled.

The chiral ligand *N*-*n*-dodecyl-L-proline was synthesized by a one-step reductive N-alkylation of the amino acid, as described in the literature [12], and purified with silica gel column chromatography. The typical experimental procedure is briefly described here. To solution of 50 mmol of L-proline (5.75 g) and 35 mmol of sodium cyanoborohydride (2.2 g NaBH_3CN) in 75 ml of methanol, 55 mmol of *n*-dodecanal (10.14 g) was added over a 20 min period at room temperature (25°C). The reaction mixture was stirred for 18 h at room temperature. The solvent was evaporated under reduced pressure and the residue was subjected to a silica gel column chromatography to purify the chiral ligand using the gradient mixture of chloroform : methanol (8:0 to 8:2) as an eluent, yielding 12.2 g of white wax. The chemical structure was determined by ^1H NMR. The residue was spotted on silica gel TLC plates and developed with chloroform : methanol (1:1, v/v). The visual detection was done by concentrated sulfuric acid vapor. The experimental results showed that R_f values of proline, *N*-*n*-dodecyl-L-proline and *n*-dodecanal were 0.31, 0.65 and over 0.95, respectively. The silica gel column chromatography was needed to remove a small amount of *n*-decanal in the residue.

2.3. Enantioselective Liquid-Liquid Extraction Experiments

The enantioselective liquid-liquid extraction based on chiral ligand exchange was conducted to determine the distribution ratio of mandelic acid and to investigate the effects of influence factors on distribution ratio and enantioseparation factor.

The biphasic solvent system was prepared as the following three steps: (1) 200 mL each of *n*-butanol and water was delivered into a 500 mL capacity of the separatory funnel. Then the content was shaken vigorously for several minutes and allowed it to equilibrate. After clear two layers were formed, two phases were separated and labelled as *n*-butanol-saturated water (S_1) and the water-saturated *n*-butanol (S_2); (2) preparation of the aqueous phase: 2.0 mg of cupric acetate monohydrate (0.02 mmol L^{-1}) and 76.1 mg of mandelic acid (10 mmol L^{-1}) were dissolved in 50 mL of S_1 ; (3) preparation of the organic phase: 0.7087 g of chiral ligand *N*-*n*-dodecyl-L-proline was dissolved in 50 mL of S_2 (50 mmol L^{-1}) and 0.2496 g of cupric acetate monohydrate was dissolved in 50 mL of S_1 (25 mmol L^{-1}), and then these two liquids were transferred into a 250 mL capacity separatory funnel and shaken vigorously for several minutes. The lower aqueous phase was abandoned after equilibration and the upper organic phase was used as the organic phase for the biphasic solvent system. The optimization of influence factors, including pH value, concentration of chiral ligand and transition metal ion were conducted during the step (3).

The equilibrium experiments were performed in 10 mL of glass-stoppered tube. Equal volumes (each 2.0 mL) of the above prepared organic and aqueous phases were delivered

into a stoppered glass tube in a water bath (20 min) at a constant temperature followed by vigorous shaking for 10 min to reach equilibrium. After phase separation, the concentration of mandelic acid enantiomers in the aqueous phase was determined by HPLC, while the concentration in the organic phase was calculated by the subtraction method. The distribution ratio (D) of the enantiomers of mandelic acid was expressed as the total concentration of enantiomers in the upper phase divided by the total concentration of enantiomers in the lower phase.

2.4. Preparation of HSCCC Solvent Systems and Sample Solutions

The biphasic solvent systems for CCC separation were prepared according to the following procedures:

(1) *n*-butanol and 200 mL of water were delivered into a 500 mL capacity separatory funnel followed by vigorous shaking for several minutes to equilibrate. After separation of the two phases the lower phase, the *n*-butanol saturated water (S_1) and the upper phase, the water saturated *n*-butanol (S_2) were obtained; (2) preparation of the aqueous phase for the biphasic solvent system: 4.0 mg of cupric acetate monohydrate (0.02 mmol L^{-1}) was dissolved in 100 mL of S_1 ; (3) preparation of the organic phase for the biphasic solvent system: 1.417 g of chiral ligand *N*-*n*-dodecyl-L-proline was dissolved in 100 mL of S_2 (50 mmol L^{-1}) and 0.499 g of cupric acetate monohydrate was dissolved in 100 mL of S_1 (25 mmol L^{-1} , pH at 5.67), and they were transferred into a 500 mL capacity separatory funnel and shaken vigorously for several minutes. The low aqueous phase was abandoned after equilibration and the organic phase was used as the upper organic phase; (4) the aqueous phase from step (2) and the organic phase from step (3) were thoroughly equilibrated in a separatory funnel, and the two phases were separated shortly before use.

The sample solutions were prepared as follows: 2.0 mg of aromatic α -hydroxyl acids was dissolved in 1.0 mL of the aqueous mobile phase, which was prepared from step (4).

2.5. HSCCC Procedure

All HSCCC separation was conducted in a head-to-tail elution mode. The enantioseparation was initiated by filling the column with the organic stationary phase. Then, the aqueous mobile phase was pumped into the column at 0.3 mL min^{-1} while the column was rotated at 1800 rpm. Each sample solution was injected after the hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet.

2.6. Chiral Analytical Method by HPLC

The chiral analysis of aromatic α -hydroxyl acids was performed by HPLC with a chiral mobile phase additive using a Shimpak CLC-ODS ($150 \times 4.6 \text{ mm i.d.}$, $5 \mu\text{m}$) column. For mandelic acid and 2-chloromandelic acid separations, the mobile phase was water-methanol (85:15, v/v) solution containing 6 mmol L^{-1} L-phenylalanine and 3 mmol L^{-1} cupric sulfate with a flow rate of 0.6 mL min^{-1} . For the separations of 4-hydroxy-3-methoxymandelic acid, 4-methoxymandelic acid, 3-chloromandelic acid, 4-bromomandelic acid, α -methylmandelic acid, α -cyclopentylmandelic acid and α -cyclohexylmandelic acid, the mobile phase was a mixture of acetonitrile and 0.1 M phosphate buffer (pH 2.68 adjusted

with phosphoric acid) containing 20 mM hydroxypropyl- β -cyclodextrin or sulfonyl butyl ether- β -cyclodextrin with a flow rate of 0.6 mL min⁻¹. The chromatographic separation conditions in detail could be found in our recent work [13]. The mobile phase was filtered through a 0.45 μ m filter and sonicated for 20 min prior to use. The wave length of the UV detector was set at 220 nm for all the analytes. The column temperature was set at 25°C during separation unless otherwise specified.

Chiral analysis of 4-hydroxymandelic acid enantiomers by HPLC was not available. The optical activities of 4-hydroxymandelic acid enantiomers of the chiral ligand exchange HSCCC fractions were determined by an automatic polarimeter Autopol I (Rudolph Research Analytical, USA).

3. Results and Discussion

3.1. Separation Mechanism for Chiral Ligand Exchange HSCCC

Chiral ligand exchange HSCCC is based on the ability of a chiral ligand (LiH) solubilized in the organic stationary phase through complexation with a transition metal ion (Cu²⁺) to preferentially extract one of the enantiomers (En) through the formation of a ternary Li⁻:Cu²⁺:En⁻ electroneutral complex [14]. Highly alkylated ligands usually partition exclusively to the organic phase of a two-phase solvent system, and its solubilization requires that the free ligand (LiH) should be in the neutral form. In order to provide a better understanding of this enantioseparation mechanism, this enantioseparation mechanism may be divided into two stages. Fig. 2, stage one (upper diagram) schematically illustrates the chemodynamic mechanisms taking place in a separatory funnel where two phases are arbitrarily separated: the organic stationary phase containing the chiral ligand (LiH) in the upper portion and the aqueous mobile phase added with transition metal ion (Cu²⁺) in the lower portion. In this first stage, a transition metal ion Cu²⁺ is extracted into the organic phase to form *bis*-binary complex Li⁻:Cu²⁺:Li⁻ by releasing two protons into the aqueous phase. In this stage the formation of binary complex in the organic phase is critically affected with pH value in the aqueous phase since protonation and deprotonation take place at the interface. This first stage is completed by the liquid-liquid extraction procedure, as described in section 2.4. Fig. 2 stage two (lower diagram) shows the chiral separation mechanism occurring in the HSCCC separation column. In this second stage, chiral ligand exchange takes place between the binary complex Li⁻:Cu²⁺:Li⁻ and enantiomer En⁻ of racemate in the aqueous phase, resulting in the formation of neutral ternary complex Li⁻:Cu²⁺:En⁻ in the organic phase. In this stage, the diastereoisomeric complex was formed with homo-chiral ternary complex, i.e., L-Li⁻:Cu²⁺:L-En⁻, and hetero-chiral ternary complex (L-Li⁻:Cu²⁺:D-En⁻), in which the enantiselectivity (α) was achieved if the stabilities of these two diastereoisomers differ. The binary complex En⁻:Cu²⁺:En⁻ could also formed in the aqueous phase in the second stage, but this formation might be neglected since the concentration of transition metal ion was very low in the second stage. HSCCC enantioseparation is completed in this second stage.

As shown in Fig. 2, during the first stage (liquid-liquid extraction), a molecule of transition metal ion in the lower aqueous phase transferred into the upper phase to form a binary

complex Li_2Cu and at the same time, two proton molecules moved to the lower phase from the upper phase. The formation constant k_{f1} for binary complex may be expressed as:

$$k_{f1} = \frac{[Li_2Cu]_{org}[H^+]_{aq}^2}{[LiH]_{org}^2[Cu^{2+}]_{aq}} \quad (1)$$

And the distribution ratio for transition metal ion is:

$$k_{Cu} = \frac{[Li_2Cu]_{org}}{[Cu^{2+}]_{aq}} \quad (2)$$

Combining equation (1) and (2) establishes the following equation for stage one:

$$\log k_{Cu} = \log k_{f1} + 2(\log [LiH]_{org} + pH_{aq}) \quad (3)$$

Equation (3) allows us to regress $\log k_{f1}$ from the intercept of a plot of $\log k_{Cu}$ vs. $\log [LiH]_{org} + pH_{aq}$. As for the second stage (chemodynamic equilibrium inside of the separation column):

Partition coefficient K_D for enantiomers:

$$K_D = \frac{[EnH_{\pm}]_{org}}{[EnH_{\pm}]_{aq}} \quad (4)$$

The dissociation constant for enantiomers:

$$k_a = \frac{[En^{-\pm}]_{aq}[H^+]_{aq}}{[EnH_{\pm}]_{aq}} \quad (5)$$

Formation constant for binary complex (k_{f1}) and ternary complex ($k_{f2\pm}$):

$$k_{f2\pm} = \frac{[Li-Cu-En_{\pm}]_{org}[LiH]_{org}}{[Li_2Cu]_{org}[EnH_{\pm}]_{org}} \quad (6)$$

Distribution ratio (D) for enantiomers could be expressed as:

$$D_{\pm} = \frac{[EnH_{\pm}]_{org} + [Li-Cu-En_{\pm}]_{org}}{[EnH_{\pm}]_{aq} + [En^{-\pm}]_{aq}} \quad (7)$$

Combining Eqs. (1) and (4)–(7), the distribution ratio D for enantiomer could be expressed as:

$$D_{\pm} = \frac{K_D}{[H^+]_{aq}^2 + k_a [H^+]_{aq}} \left(k_{f1} k_{f2\pm} [Cu^{2+}]_{aq} [LiH]_{org} + [H^+]_{aq}^2 \right) \quad (8)$$

And the enantioseparation factor α for enantiomers enantioseparated by chiral ligand exchange countercurrent chromatography would be:

$$\alpha = \frac{k_{f1} k_{f2+} [Cu^{2+}]_{aq} [LiH]_{org} + [H^+]_{aq}^2}{k_{f1} k_{f2-} [Cu^{2+}]_{aq} [LiH]_{org} + [H^+]_{aq}^2} \quad (9)$$

As shown in equations (8) and (9), for a given two-phase solvent system at constant separation temperature, distribution ratio for enantiomers would be largely determined by partition coefficient K_D , pH value of the aqueous phase, binary and ternary complex formation constant, concentration of transition metal ion and concentration of chiral ligand in the organic phase, where as the enantioseparation factor is critically dependent on several parameters such as the difference between two ternary complex formation constants k_{f2+} and k_{f2-} , concentration of transition metal ion in the aqueous phase, concentration of chiral ligand in the organic phase, and pH value in the aqueous phase.

3.2. Optimization of Two-Phase Solvent Systems

The most important problem for enantioseparation by HSCCC is to select a biphasic solvent system along with the chiral selector with high enantio-recognition. In addition to the general requirements for solvent systems used in conventional separations, the chiral selector should be soluble only in one phase while the enantiomers could freely partition in both phases of the solvent system. Furthermore, the enantio-recognition ability of the chiral selector shouldn't be disrupted by the selected solvent system. Chiral ligand exchange HSCCC utilizes the unique configuration of transition metal complexes containing a chiral ligand to separate enantiomers. The chiral ligand and transition metal ion for a given racemate were generally referred from other techniques, such as HPLC and enantioselective liquid-liquid extractions. H. Nishizawa reported the enantioseparation of racemic mandelic acid by continuous separation method with liquid particle extractor using a solvent system of *n*-butanol : water (1:1, v/v) containing a coordination complex between the chiral ligand *N*-*n*-dodecyl-L-proline and the transition metal ion Cu^{2+} [15]. However, the solvent system with the chiral ligand exchange used in the literature was not fully exploited, e.g., the pH value of the aqueous phase greatly affects the partition coefficient of transition metal ion Cu^{2+} , which would lead to a great difference for distribution of enantiomers. However, influence of pH value was not mentioned in that study. Additionally, as shown in equation (8) and (9), distribution ratio and enantioseparation factor are critically related with pH value, concentration of chiral ligand and transition metal ion. Thus, these influence factors for the solvent system on distribution behavior of mandelic acid enantiomers were investigated in the present study as described in experimental *section 2.3*.

Influence of pH value on distribution behavior of mandelic acid enantiomers was first investigated. As discussed in the *section 3.1*, partition of Cu^{2+} into the organic phase was

mainly dependent on the pH value. The binary complex between chiral ligand and Cu^{2+} was easily formed in the organic phase when the pH in the aqueous phase was increased, which enhanced the formation of ternary complex between chiral ligand, Cu^{2+} and enantiomers. So the distribution ratio increased with the increase of pH value in the system (Fig. 2), and the enantioseparation factor also gradually increased. However, copper complex was found to be precipitated when the pH was over 6.0. Therefore, pH 5.57 was selected in our research.

Then, several different types of the organic solvent were examined. The effect of organic solvent on the distribution ratio and enantioseparation factor of enantiomers was summarized in Table 1. The chiral ligand *N*-*n*-dodecyl-L-proline couldn't dissolve in the ethyl acetate or methyl *tert*-butyl ether. Although chloroform, dichloromethane and 1, 2-dichloroethane gave the suitable distribution ratio, only very low enantioselectivities were obtained. The alcohols such as *n*-butanol and *n*-pentanol provided the higher enantioseparation factor and suitable distribution ratio. Since higher viscosity of *n*-pentanol would lead to lower retention of the stationary phase, *n*-butanol was selected for HSCCC separation.

Third, the effect of temperature on distribution ratio and enantioseparation factor was summarized in Fig. 3. The results showed that low temperature favored chiral recognition for enantioseparation of mandelic acid.

Finally, the concentrations of chiral ligand and transition metal ion were investigated, in which three experimental steps were made. First, the distribution ratio and enantioseparation factor of enantiomers were measured by changing concentration of chiral ligand 0–80 mmol L^{-1} while the concentration of Cu^{2+} was kept constant at 25 mmol L^{-1} . It was found that the enantioseparation factor reached the highest value when the concentration of chiral ligand was about 60 mmol L^{-1} (Fig. 4). Second, the distribution ratio and enantioseparation factor were investigated by changing the concentration of Cu^{2+} at 0–50 mmol L^{-1} while the concentration of chiral ligand was kept at 50 mmol L^{-1} . Fig. 5 shows that high enantioseparation factor along with suitable distribution ratio was obtained when the concentration of Cu^{2+} was over 25 mmol L^{-1} . The above results indicated that the highest enantioselectivity was achieved when the ratio of chiral ligand to Cu^{2+} was about 2:1, which agrees with the equilibrium formation of binary coordination complex in the organic phase. Thus, the final experiment was completed by changing concentration of both chiral ligand and Cu^{2+} under the constant molecular ratio of 2:1 for chiral ligand and metal ion (Fig. 6). The results showed that when the concentrations of chiral ligand and metal ion were 40–50 mmol L^{-1} and 20–25 mmol L^{-1} , respectively, a high enantioseparation factor as well as the suitable distribution ratio could be obtained for enantioseparation of mandelic acid by HSCCC

It is necessary to point out here that the concentration of the racemic sample would inevitably alter its distribution ratio and enantioseparation factor. Generally, in order to make sure that the molar concentration of the sample is much smaller than that of chiral selector, a low concentration of the racemic sample, e.g., less than 1.0 mmol mL^{-1} , was used for series of enantioselective liquid-liquid extractions for selection and optimization of the biphasic solvent systems. But, as indicated in the experimental *section 2.3*, the concentration

of mandelic acid, 10 mmol L^{-1} , was used. This high concentration was used to obtain suitable response values during the HPLC measurement. As for chiral analysis of mandelic acid by HPLC with L-phenylalanine and Cu^{2+} as a chiral mobile phase additive, serious interference of UV absorbance by L-phenylalanine was observed and little response values could be obtained for mandelic acid enantiomers if the low concentrations, e.g. $1\text{--}3 \text{ mmol mL}^{-1}$, was used for sample injection. Therefore, a relatively high concentration of the mandelic acid sample was used for the above enantioselective liquid-liquid extraction experiments.

3.3. Enantioseparation of aromatic α -hydroxyl acids by HSCCC

Though only the solvent system for mandelic acid was optimized, other aromatic α -hydroxyl acids, including 2-chloromandelic acid, 4-methoxymandelic acid, 4-hydroxymandelic acid, 4-hydroxy-3-methoxymandelic acid, 4-bromomandelic acid, 3-chloromandelic acid, α -methylmandelic acid, α -cyclohexylmandelic acid and α -cyclopentylmandelic acid were also enantioseparated by HSCCC. Among the above 10 derivatives only five of them, including mandelic acid, 2-chloromandelic acid, 4-methoxymandelic acid, 4-hydroxymandelic acid and α -methylmandelic acid were successfully enantioseparated by chiral ligand exchange HSCCC.

The racemic 4-hydroxy-3-methoxymandelic acid and 4-bromomandelic acid were eluted out without any resolutions using the same solvent system with the retention time of 70–105 min and 190–230 min, respectively; and for the racemic 3-chloromandelic acid, α -cyclohexylmandelic acid and α -cyclopentylmandelic acid, most of the enantiomers were partitioned into the upper organic phase and their retention times were over 320 min. In order to improve their solubilities in the aqueous phase of the solvent system, hydroxypropyl- β -cyclodextrin was added in the aqueous phase. 4-Bromomandelic acid could only dissolve in the aqueous phase added with cyclodextrin. However, no successful enantioseparation was achieved for this hydrophilic racemates. As for the enantioseparation of 4-hydroxy-3-methoxymandelic acid, the ligand di-(2-ethylhexyl) phosphoric acid was added in the solvent system to improve its partition into the organic phase, but no successful enantioseparation was achieved.

Fig. 7 shows the typical chromatogram for enantioseparation of mandelic acid, 2-chloromandelic acid, 4-methoxymandelic acid, 4-hydroxymandelic acid and α -methylmandelic acid by chiral ligand exchange HSCCC. The fractions collected from the enantioseparation were analyzed by analytical chiral HPLC. For the chiral analysis of fractions of 4-hydroxymandelic acid, no chiral HPLC analysis were available and the automatic polarimeter was used to determine the optical rotation for the eluates, in which the results indicated that the first peak was (–)-enantiomer and the second peak, (+)-enantiomer.

HSCCC can be easily scaled up for preparative chiral separations. But the amount of sample allowed to be injected into the CCC column should not be larger than the amount of chiral selector retained in the column, which is critically related to the molar ratio between the complex formed by chiral selector and analytes during the separation. For the present chiral ligand exchange HSCCC, the enantiorecognition process was achieved by formation of the diastereoisomeric ternary coordination complex through the ligand exchange between the

free molecule of mandelic acid enantiomer in the aqueous phase and *N-n*-dodecyl-L-proline of the binary complex in the organic phase where the molar ratio for *N-n*-dodecyl-L-proline, Cu^{2+} and enantiomer was 2:1:1, as shown in the separation mechanism. On the other hand, the amount of chiral ligand retained in the stationary phase was highly dependent on the retention of stationary phase. It was found that the retention volume for the organic stationary phase was only about 4.0 mL after hydrodynamic equilibrium was established in the separation column prior to sample injection, as calculated by the displaced stationary phase volume, and the retention volume for the organic stationary phase decreased down to about 3.0–3.5 mL after separation due to its steady carryover during the separation. So the amount of chiral ligand in the column before sample injection was about 0.10 mmol, and in this case, the amount of sample to be loaded should be limited to less than 0.10 mmol.

3.4. Comparison with Conventional Chiral Ligand Exchange HPLC

Quite a few of literatures were available about analytical enantioseparation of six aromatic α -hydroxyl acids by chiral ligand exchange HPLC using either chiral stationary phase [16–21] or chiral mobile phase additive [22–24]. But, no literature is available about chiral separation of four other racemates, including 4-methoxymandelic acid, 3-chloromandelic acid, α -cyclopentylmandelic acid and α -cyclohexylmandelic acid, by chiral ligand exchange HPLC.

Enantioseparations of the ten aromatic α -hydroxyl acids by HPLC with a chiral ligand mobile additive were also investigated in our research. A conventional ODS C_{18} column was used as the stationary phase and the mobile phase was composed of 6 mmol L^{-1} of L-amino acid and 3 mmol L^{-1} of cupric sulfate and methanol with different volume ratios. Three chiral ligand L-amino acids, including L-proline, L-alanine, and L-phenylalanine, were tested for enantioseparation of the ten aromatic α -hydroxyl acids. Experimental results showed that only three of them, mandelic acid, 2-chloromandelic acid and α -methylmandelic acid, could be successfully enantioseparated by HPLC with L-phenylalanine as the chiral ligand. No successful enantioseparation of aromatic α -hydroxyl acids was achieved by HPLC when either L-proline or L-alanine was used as the chiral ligand. The enantiomers of 4-hydroxymandelic acid and 4-hydroxy-3-methoxymandelic acid were eluted out within several minutes without resolutions with the mobile phase consisting of aqueous solution : methanol at various volume ratios containing 6 mmol L^{-1} of L-phenylalanine and 3 mmol L^{-1} of cupric sulfate. For other racemates such as 4-methoxymandelic acid, 3-chloromandelic acid, 4-bromomandelic acid, α -cyclopentylmandelic acid and α -cyclohexylmandelic acid, they are highly hydrophobic and couldn't be dissolved in the mobile phase of aqueous solution : methanol (80:20, v/v). Though they could dissolve in the mobile phase with the proportion of 40:60, no successful enantioseparations were obtained.

Table 2 shows the comparison of HSCCC and HPLC in enantioseparation of ten racemates. Though enantioseparation of 4-hydroxymandelic acid and 4-methoxymandelic acid by chiral ligand exchange HSCCC could be successfully applied, they were difficult to be enantioseparated by reverse phase HPLC with chiral ligand used as a mobile phase additive. The chiral stationary phase (chiral ligand column) was needed for enantioseparation of 4-

hydroxymandelic acid by HPLC. However, as shown in Fig. 7, successful enantioseparation of 4-hydroxymandelic acid was achieved by analytical HSCCC.

4. Conclusion

Chiral ligand exchange HSCCC was successfully applied to enantioseparation of five aromatic α -hydroxyl acids with *N*-*n*-dodecyl-L-proline as a chiral ligand in the organic phase and Cu^{2+} as a transition metal ion in the aqueous phase. The separation mechanism for a typical chiral ligand exchange HSCCC was proposed, in which distribution ratio and enantioseparation factor were deduced by the equilibrium correlations. Distribution ratio and enantioseparation factor were critically dependent on the concentration of chiral ligand, transition metal ion and pH value of the solvent system. Optimization of the two-phase solvent system for racemic mandelic acid was established. Our results show that chiral ligand exchange HSCCC may be considered as an efficient alternative chiral chromatographic technique for enantioseparations.

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Fig. 1. chemical structures of aromatic α -hydroxy acids and chiral ligand used in the present research

Fig. 2. Schematic diagram of speciation reactions in a chiral ligand exchange two-phase solvent system (stage one) and chemodynamic equilibrium between racemates ($\text{EnH}_{\pm}$) and chiral ligand (LiH) in the separation column (stage two). $[\text{EnH}_{\pm}]$: the concentration of both (+) and (−) isomers, $[\text{Li-Cu-En}_{\pm}]$: concentration of diastereoisomeric ternary complex, K_D : partition coefficient, k_f : the formation constant for binary complex and ternary complex, k_a : dissociation constant for enantiomers.

Fig. 2. Influence of pH on the distribution ratio (D) and enantioseparation factor (α) for mandelic acid enantiomers. Solvent systems: the organic phase containing 0.05 mol L^{-1} *N-n*-dodecyl-L-proline was prepared by step (3) of section 2.3, but it was equilibrated with 0.1 mol L^{-1} acetate buffer (pH 4.47, 5.57, 6.49) or phosphate buffer (pH 2.64) containing 25 mmol L^{-1} of cupric acetate monohydrate; the aqueous phase containing 0.02 mmol L^{-1} cupric acetate monohydrate and 10 mmol L^{-1} of mandelic acid was prepared by step (2); Equilibration temperature: 5°C .

Fig. 3. Influence of temperature on the distribution ratio (D) and enantioseparation factor (α) for mandelic acid enantiomers. Solvent systems: the aqueous phase and organic phase were prepared by step (2) and (3) of section 2.3.

Fig. 4. Effects of concentration of chiral ligand on the distribution ratio (D) and enantioseparation factor (α) for mandelic acid enantiomers. Solvent systems: the organic phase containing different concentration of *N-n*-dodecyl-L-proline was prepared from step (3) of section 2.3; the aqueous phase was prepared from step (2) containing 0.02 mmol L⁻¹ cupric acetate monohydrate and 10 mmol L⁻¹ of mandelic acid; Equilibration temperature: 5°C.

Fig. 5.

Effects of concentration of Cu^{2+} on the distribution ratio (D) and enantioselectivity factor (α) for mandelic acid enantiomers. Solvent systems: the organic phase containing 0.05 mol L^{-1} of *N*-*n*-dodecyl-L-proline was prepared from step (3) of section 2.3, and it was equilibrated with different concentration of cupric acetate monohydrate; the aqueous phase was prepared from step (2) containing 0.02 mmol L^{-1} cupric acetate monohydrate and 10 mmol L^{-1} of mandelic acid. Equilibration temperature: 5°C .

Fig. 6. Effects of concentration of chiral ligand and Cu^{2+} at constant ratio of 2:1 on the distribution ratio (D) and enantioseparation factor (α) for mandelic acid enantiomers. Solvent systems: the organic phase containing different concentration of *N*-*n*-dodecyl-L-proline was prepared from step (3) of section 2.3, and it was equilibrated with aqueous phase containing different concentration of cupric acetate monohydrate; the aqueous phase was prepared from step (2) containing 0.02 mmol L^{-1} cupric acetate monohydrate and 10 mmol L^{-1} of mandelic acid. Equilibration temperature 5°C .

Fig. 7.

Chromatograms of enantioseparation of racemic aromatic α -hydroxyl acids by chiral ligand exchange HSCCC. Solvent system: see experimental *section 2.4*; stationary phase: upper organic phase; mobile phase: lower aqueous phase; sample solution: 2.0 mg of racemates dissolved in 1 mL of the mobile phase; flow rate: 0.3 mL/min; revolution: 1800 rpm; column temperature: 10°C; stationary phase retention: 15–20 %.

Table 1Effect of organic solvent on the distribution ratio (D) and enantioseparation factor (α) of mandelic acid

Organic solvent	D_+	D_-	α
<i>n</i> -Butanol	5.828	3.591	1.623
<i>n</i> -Pentanol	4.493	2.754	1.631
Chloroform	4.793	5.498	1.147
Dichloromethane	3.417	3.460	1.013
1,2-Dichloroethane	2.239	2.242	1.002
Ethyl acetate	-	-	-
Methyl <i>tert</i> -butyl ether	-	-	-

Solvent system: the aqueous phase containing 0.02 mmol L cupric acetate monohydrate and 10 mmol L⁻¹ of mandelic acid was prepared from step (2) of section 2.3; the organic phase was prepared from step (3) with different organic solvent; Equilibration temperature 5°C.

Table 2

Enantioseparation of the ten racemates by chiral ligand exchange countercurrent chromatography and high performance liquid chromatography

Racemate	High speed countercurrent chromatography	Liquid chromatography in our lab	Liquid chromatography in the literatures
mandelic acid	butanol-water (1:1, v/v) (N-n-dodecyl-L-proline and Cu ²⁺)	CLEMP (L-phenylalanine and Cu ²⁺)	CLESP [18–20]; CLEMP (L-phenylalanine and Cu ²⁺) [23] and (N,N-di-propyl-L-alanine, N,N-dimethyl-L-valine as chiral ligand and RP8 stationary phase) [24]
2-chloromandelic acid	butanol-water (1:1, v/v) (N-n-dodecyl-L-proline and Cu ²⁺)	CLEMP (L-phenylalanine and Cu ²⁺)	CLEMP (L-leucine and Zn ²⁺) [22]
4-methoxymandelic acid	butanol-water (1:1, v/v) (N-n-dodecyl-L-proline and Cu ²⁺)	N/A	N/A
4-hydroxymandelic acid	butanol-water (1:1, v/v) (N-n-dodecyl-L-proline and Cu ²⁺)	N/A	CLESP [17,19,20]; CLEMP with N,N-dimethyl-L-phenylalanine as chiral ligand and C ₁₈ stationary phase [23] or N,N-di-propyl-L-alanine, N,N-dimethyl-L-valine as chiral ligand and RP8 stationary phase [24]
α-methylmandelic acid	butanol-water (1:1, v/v) (N-n-dodecyl-L-proline and Cu ²⁺)	CLEMP (L-phenylalanine and Cu ²⁺)	CLESP [18,19]; CLEMP (L-phenylalanine and Cu ²⁺) [23] and (N,N-di-propyl-L-alanine, N,N-dimethyl-L-valine as chiral ligand and C ₁₈ stationary phase) [24]
4-hydroxy-3-methoxy-mandelic acid	N/A	N/A	CLESP [20]
3-chloromandelic acid	N/A	N/A	N/A
4-bromomandelic acid	N/A	N/A	CLESP [16]
α-cyclopentylmandelic acid	N/A	N/A	N/A
α-cyclohexylmandelic acid	N/A	N/A	N/A

Note: the stationary phase for chiral ligand exchange HPLC used in our research was a conventional ODS C₁₈ column.

CLESP: chiral ligand exchange stationary phase; CLEMP: chiral ligand exchange mobile phase

N/A: not available