Published in final edited form as:

J Chromatogr A. 2010 April 30; 1217(18): 3044–3052. doi:10.1016/j.chroma.2010.02.077.

Chiral separation of α -cyclohexylmandelic acid enantiomers by high-speed counter-current chromatography with biphasic recognition

Shengqiang Tong

Department of Chemical and Biochemical Engineering, Zhejiang University, Hangzhou, 310027, China

Abstract

This work concentrates on a novel chiral separation technology named biphasic recognition applied to resolution of α -cyclohexylmandelic acid enantiomers by high-speed counter-current chromatography (HSCCC). The biphasic chiral recognition HSCCC was performed by adding lipophilic (–)-2-ethylhexyl tartrate in the organic stationary phase and hydrophilic hydroxypropyl- β -cyclodextrin in the aqueous mobile phase, which preferentially recognized the (–)-enantiomer and (+)-enantiomer, respectively. The two-phase solvent system composed of *n*-hexane-methyl *tert*-butyl ether-water (9:1:10, v/v/v) with the above chiral selectors was selected according to the partition coefficient and separation factor of the target enantiomers. Various parameters involved in the chiral separation were investigated, namely the types of the chiral selector (CS); the concentration of each chiral selector; pH of the mobile phase; and the separation temperature. The mechanism involved in this biphasic recognition chiral separation by HSCCC was discussed. Langmuirian isotherm was employed to estimate the loading limits for each chiral selector. The overall experimental results show that the HSCCC separation of enantiomer based on biphasic recognition is much more efficient than the traditional monophasic recognition chiral separation, since it utilizes the cooperation of both lipophilic and hydrophilic chiral selectors.

Keywords

Chiral separation; High-speed counter-current chromatography; Biphasic chiral recognition; α -Cyclohexylmandelic acid; Optical activity

1. Introduction

Chromatographic resolution of enantiomers has become increasingly important for both qualitative and quantitative analyses applied for controlling the enantioselectivity of a reaction or separation of racemic mixtures [1]. High-performance liquid chromatography (HPLC) has been most frequently used for chiral separation and more than one hundred chiral stationary phases are commercially available for analytical-scale separations [2]. In the HPLC technique, the chiral selector is chemically bonded to a solid support that serves as a stationary phase. Manufacturing such a chiral stationary phase, however, requires a series of time-consuming complicated processes and the resulting columns are very expensive, particularly for preparative-scale separations [3].

Counter-current chromatography (CCC) is a liquid-liquid chromatographic technique, without a solid support in which the stationary and the mobile phases are constituted by two immiscible liquids or solutions [4]. Therefore, it is an excellent alternative since the column requires no solid support and can be used to separate a variety of enantiomers simply by adding a suitable

chiral selector to the stationary liquid phase. The CCC technique is very cost-effective in chiral separations because the method permits repetitive use of the same column for a variety of chiral separations with a high sample loading capacity. However, in the past 26 years only 25 papers on chiral CCC separation have been published due to the difficulty of finding the optimum chiral selectors. Three chiral selectors including N-dodecanoyl-L-proline-3, 5-dimethylanilide, sulfated β -cyclodextrin and vancomycin have proved most efficient in chiral CCC or centrifugal partition chromatography [5]. Under most circumstances, the chiral CCC technique was applied in separation of ($\pm$)-DNB-amino acids [1-3,9,11], racemates of drugs and their metabolites [6-10].

Generally, chiral CCC separation needs a suitable hydrophilic or lipophilic chiral selector which is dissolved either in the stationary phase or in the mobile phase for monophasic recognition, and chiral separations are very often characterized by a separation factor, α , in the 1.05 to 1.3 range [5]. Although CCC is a powerful preparative techniques with its high capacity, low cost of stationary phases and low solvent consumption, it yields low efficiency compared to HPLC. For this reason, the method requires a separation factor greater than 1.5 to completely resolve two peaks. The major difficulty is to find chiral selectors that are highly selective in the liquid phase of the two-phase solvent system that does not affect their selectivity and retains the capacity to elute chiral isomers of interest. Since the CCC community constitutes a small group, new ideas and new chiral selectors will generally come from other separation techniques [5]. Taking the inspiration from literature [12], a novel chiral CCC separation method called biphasic chiral recognition is developed by adding lipophilic chiral selector in the stationary phase and hydrophilic one in the mobile phase which preferentially recognize the (–)-enantiomer and (+)-enantiomer, respectively. It was found that this novel method remarkably improves the separation factor.

In the present studies, (–)-2-ethylhexyl tartrate was used as lipophilic chiral selector in the stationary phase for recognition of the (–)-enantiomer while hydroxypropyl- β -cyclodextrin (HP- β -CD) as hydrophilic chiral selector in the mobile phase of recognition of the (+)-enantiomer. Various parameters involved in the chiral separation of α -cyclohexylmandelic acid were investigated, namely the types of the chiral selector, the concentration of the two chiral selectors, pH values of the mobile phase and the temperature. Langmuirian isotherm and sample size were determined under the given values of the two CSs concentration.

α -Cyclohexylmandelic acid (CHMA) is a significant chiral drug precursor used for the synthesis of chiral drugs such as oxybutynin which is the principal drug for curing urinary incontinence and has a wide market. Since the (+)-enantiomer has a better drug effect with less side effects than the racemic mixture, it is necessary to either resolve the racemic mixture or esterify the chiral precursor (+)-CHMA in order to obtain (+)-oxybutynin; the latter of which can reduce the cost greatly [12].

2. Experimental Section

2.1 Apparatus

Two different models of HSCCC apparatus were used in the present study: TBE-20A analytical and TBE-300A preparative multilayer coil planet centrifuges (Shanghai Tauto Biotechnology, Shanghai, China) each equipped with a set of three multilayer coils. The TBE-20A analytical column consists of 0.8 mm ID PTFE tubing with a total capacity of 20 mL while the TBE300A preparative column consists of 1.6 mm ID PTFE tubing with a total capacity of 260 mL. The β values of the analytical and preparative columns ranged from 0.60 to 0.78 and 0.46 to 0.73, respectively ($\beta=r/R$, $R=4.5$ cm for analytical columns and 6.5 cm for preparative ones, where r is the distance from the coil to the holder shaft, and R , the revolution radius or the distance between the holder shaft and central axis of the centrifuge).. The revolution speed of the

apparatus can be regulated with a speed controller in the range from 0 to 2000 rpm for the TBE-20A analytical centrifuge, and from 0 to 1000 rpm for the TBE-300A preparative centrifuge where the optimum speed of 1820 rpm was used for the analytical columns and 850 rpm for preparative columns. In both CCC centrifuges separation columns were installed in a vessel that maintains column temperature at 8 °C by a Model HX-1050 constant-temperature controller (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China). Manual sample injection valves with a 1.0 mL loop for analytical apparatus and a 20.0 mL loop for preparative one were used to introduce the sample into the column. The solvents were pumped into the column with a model TBP-1002 constant-flow pump for analytical separation and a model TBP-50A constant-flow pump for preparative separation (Shanghai Tauto Biotechnology, Shanghai, China). Continuous monitoring of the effluent was achieved with a model 8823B UV detector (Beijing BINTA Instrument Technology Co., Ltd., Beijing, China) at 254 nm, and SEPU3000 workstation (Hangzhou PuHui Technology, Hangzhou, China) was employed to record the chromatogram. Eluate was collected with a Model BSZ-160 fraction collector (Shanghai Huxi Tech, Shanghai, China).

The pH value was manually determined with a portable Delta 320-s pH meter (Mettler–Toledo, Greifensee, Switzerland). The high-performance liquid chromatography (HPLC) used was a CLASS-VP Ver.6.1 system (Shimadzu, Japan) comprised of a Shimadzu SPD10Avp UV detector, a Shimadzu LC-10ATvp Multisolvent Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, and a CLASS-VP Ver.6.1 workstation.

2.2 Reagents

HP- β -CD was purchased from Xinda Fine Chemical & Co. Inc., Shandong, China. D-(–)-tartrate acid was purchased from Shanghai Xinpu Chemical Factory, Shanghai, China. *n*-butanol, *isobutanol*, *n*-pentanol, *n*-hexanol, *n*-octanol, 2-ethyl hexanol, *n*-dodecanol, toluene and *p*-toluenesulfonic acid were purchased from Hangzhou HuiPu Chemical, Hangzhou, China. α -Cyclohexyl-mandelic acid (CHMA) was purchased from Guangde Keyuan Chemical Co., Ltd, Guangde, China. All organic solvents used for CCC separations were of analytical grade. Acetonitrile (AcN) and ethanol used for HPLC analysis were of chromatographic grade. Methyl *tert*.-butyl ether (MtBE) was redistilled before use.

2.3. Synthesis of (–)-Tartaric Acid Derivatives

(–)-2-Ethylhexyl tartrate was prepared as follows: powdered (–)-tartaric acid (0.05 mol), 2-ethylhexanol (0.14 mol) and 4-toluenesulphonic acid (0.0015 mol) were refluxed in anhydrous toluene (30mL) with continual removal of water using a Dean and Stark apparatus until the theoretical amount of water had been removed. The mixture was cooled and the toluene solution washed with 5% sodium hydrogen carbonate solution (3×50 mL) followed by water (3 × 50 mL). The organic phase was dried over anhydrous magnesium sulfate. The toluene and 2-ethylhexanol were removed by distillation under reduced pressure to give the product (95% yield). (–)-2-ethylhexyl tartrate was a colorless liquid under air atmosphere. ¹H NMR (CDCl₃) δ 4.524 (2H, s), δ 4.150 (4H, m), δ 1.633 (2H, *br.*), δ 1.390 (4H, m), δ 1.310 (12H, m) and δ 0.906 (12H, t). *n*-Butyl tartrate, *isobutyl* tartrate, *n*-pentyl tartrate, *n*-hexyl tartrate, *n*-octyl tartrate and *n*-dodecyl tartrate were also prepared by the same procedure and their structures were confirmed by ¹H NMR. Among those compounds *n*-hexyl tartrate and 2-ethylhexyl tartrate were colorless liquid at air atmosphere while the other compounds were all solids.

2.4. Effects of Various Parameters on Separation Factor

HP- β -CD was used as the chiral selector in the aqueous phase. The aqueous phases were prepared by dissolving HP- β -CD and CHMA enantiomer in a 0.1 mol L^{–1} phosphate salt buffer solution. (–)-2-Ethylhexyl tartrate used as the chiral selector and dissolved in the organic phases

composed of *n*-hexane-methyl *tert*.-butyl ether (9:1, v/v). The equilibrium experiments were performed in 10 mL glass-stoppered tube. Equal volumes (each 2.0 mL) of the organic and aqueous phases were placed in a stoppered glass tube and shaken vigorously for 10 min before being kept in a water bath (30 min) at a constant temperature to reach equilibrium. After phase separation, the concentrations of α -CHMA in the aqueous and organic phases were determined by HPLC..

2.5. Preparation of CCC Solvent Systems and Sample Solutions

Solvent systems consisting of *n*-hexane-MtBE-water (9:1:10, v/v/v) was used. The solvent mixture was thoroughly equilibrated in a separatory funnel, and the two phases were separated shortly before use. A given amount of the CS was added to the organic stationary phase. The sample solutions were prepared as follows: 3.5-22 mg of α -cyclohexylmandelic acid enantiomer was dissolved in the 1 mL of the upper phase with the chiral selector for the analytical separations. and 365 mg of α -cyclohexylmandelic acid enantiomer was dissolved in the 20 mL of the upper phase with the chiral selector for the preparative separations.

2.6. General CCC Procedure

Both of analytical and preparative separations were initiated by filling the column with the organic stationary phase. The aqueous mobile phases were pumped into the column while the column was rotated at 1820 rpm for analytical separations and 850 rpm for preparative separations. The absorbance of the effluent was continuously monitored at 254 nm. The optical activity of the CCC fractions was assessed using the polarimeter.

2.7. Recovery of Solutes from Chiral CCC Fractions

The collected mobile phase fractions containing the separated enantiomers were acidified with a small volume of concentrated hydrochloric acid and extracted five times with diethyl ether. The combined organic layers were dried with anhydrous magnesium sulfate and filtered, and the solvent was evaporated. The residue of the organic layers was spotted on silica gel plates and developed with chloroform: methanol (15:1, v/v). The visual detections were done by concentrated sulfuric acid vapor. The experimental result showed that R_f value of ($\pm$)-enantiomer spots on the TLC was 0.41, R_f values of ($-$)-2-ethylhexyl tartrate and hydrophilic CS HP- β -CD were 0.82 and 0.05, respectively. In order to purify the CHMA enantiomers, the residue was further subjected to the silica gel column chromatography with gradient elution (chloroform : methanol from 15:0 to 15 :1) to remove the small amount of lipophilic CS ($-$)-2-ethylhexyl tartrate and hydrophilic CS HP- β -CD.

2.8. Analytical Method

The quantification of α -cyclohexylmandelic acid enantiomer was performed by HPLC using a UV DETECTOR at the UV wavelength of 220 nm. The column was YMC-Pack ODS-A, 5 μ m particle size of the packing material, 250 mm $\times$ 4.6 mm I.D. (YMC Co., Ltd., Kyoto, Japan). The mobile phase was 0.075 molL⁻¹ KH₂PO₄ aqueous solution: alcohol: acetonitrile (65:20:15) containing 9.5 mmolL⁻¹ β -CD at a flow of 0.6 mLmin⁻¹. The retention time of the (+)-CHMA was less than that of the ($-$)-CHMA.

3. Results and Discussion

3.1. Selection of Two-Phase Solvent Systems and Chiral Selectors

Enantioselective resolution of α -CHMA by biphasic recognition chiral extraction was conducted by the two-phase solvent system composed of 1, 2-dichloroethane-0.1molL⁻¹ phosphate salt buffer solution (1:1, v/v) [12]. The lipophilic chiral extractant ($-$)-*isobutyl* tartrate was dissolved in the organic phase at 0.2 molL⁻¹ and the hydrophilic chiral extractant

HP- β -CD in the aqueous phase at 0.1 molL^{-1} . Since the above solvent system gave the satisfactory separation factor for α -cyclohexylmandelic acid under the temperature 5°C , we tried to separate α -cyclohexylmandelic acid enantiomers by CCC using the above solvent system. However, CCC separation had been unsuccessful mainly due to its high degree of emulsification of the solvent system under 5°C . So further efforts were needed to achieve the separation of enantiomers [11]. Liquid-liquid extraction experiments were necessary to find suitable solvent systems, which should satisfy the following requirements. First, each CS should be soluble in only one of the two phases. For a two-phase aqueous/organic system, the selector should be either highly lipophilic to remain entirely in the organic phase or highly hydrophilic to remain entirely in the aqueous phase. Second, the racemic mixtures should be easily soluble in both phases. Third, the optimum distribution ratio is about 1 for CCC separation [13]. High solute distribution in the stationary phase will lead to long retention times whereas, too high distribution of the compound in the mobile phase will reduce the chromatographic efficiency of enantioseparation due to the reduced time for interaction between enantiomers and CS.

An initial procedure was designed to assess the potential enantioselectivity of CSs in various solvent systems by means of extraction experiment. A wide range of binary, ternary and quaternary two-phase solvent systems was evaluated. First, their readiness for forming emulsions was taken into account. The solvent systems that separated rapidly were subsequently tested with the chiral selector to measure their distribution between the two phases. The two-phase solvent systems composed of chloroform- 0.1 molL^{-1} phosphate salt buffer solution with $\text{pH}=2.68$ (1:1, v/v), *n*-butanol- 0.1 molL^{-1} phosphate salt buffer solution with $\text{pH}=2.68$ (1:1, v/v), *n*-hexane- 0.1 molL^{-1} phosphate salt buffer solution with $\text{pH}=2.68$ (1:1, v/v), MtBE- 0.1 molL^{-1} phosphate salt buffer solution with $\text{pH}=2.68$ (1:1, v/v), MtBE-acetonitrile- 0.1 molL^{-1} phosphate salt buffer solution with $\text{pH}=2.68$ (2:2:3, v/v/v) and *n*-hexane-ethyl acetate-methanol- 0.1 molL^{-1} phosphate salt buffer solution with $\text{pH}=2.68$ (1:1:1:1, v/v/v/v) were tested by adding the lipophilic CS 0.2 molL^{-1} (–)-*isobutyl* tartrate and the hydrophilic CS 0.1 molL^{-1} HP- β -CD. Among those solvent systems only *n*-hexane- 0.1 molL^{-1} phosphate salt buffer solution with $\text{pH}=2.68$ (1:1, v/v) gave the suitable partition ratio for the (+)-enantiomer and (–)-enantiomer. However, poor solubility of the lipophilic chiral selector (–)-*isobutyl* tartrate and the racemic mixtures in the *n*-hexane was the problem in this system. When a small amount MtBE was added to this system, solubility of racemic mixtures was greatly improved but solubility of (–)-*isobutyl* tartrate still remained poor at 5°C . The two-phase solvent system composed of *n*-hexane-MtBE- 0.1 molL^{-1} phosphate salt buffer solution with $\text{pH}=2.68$ (9:1:10., v/v/v) gave $K_R=1.886$ and $K_S=1.171$ and the separation factor α was 1.611, which was quite lower than the separation factor $\alpha=2.49$ obtained with the system 2-dichloroethane- 0.1 molL^{-1} phosphate salt buffer solution with $\text{pH}=2.68$ (1:1, v/v) in the literature [12]. The above results showed that the system had a lower chiral recognition if the lipophilic chiral selector (–)-*isobutyl* tartrate has low solubility in the organic phases. Therefore, six of other tartrate compounds were prepared according to literature [14], including (–)-*n*-butyl tartrate, (–)-*n*-pentyl tartrate, (–)-*n*-hexyl tartrate, (–)-*n*-octyl tartrate, (–)-2-ethylhexyl tartrate and (–)-*n*-dodecyl tartrate. All the other lipophilic CSs except (–)-*n*-dodecyl tartrate had improved solubility in the organic phase of the solvent system. Especially, (–)-*n*-hexyl tartrate and (–)-2-ethylhexyl tartrate were liquid and they are completely miscible with the organic phase at any proportion. A series of liquid-liquid extraction experiments under 8°C demonstrated that when (–)-2-ethylhexyl tartrate was used as the lipophilic CS in the organic phases of the solvent system, it produced a suitable distribution ratio for both (–)-enantiomer ($D_- = 3.885$) and (+)-enantiomer ($D_+ = 1.933$) with a high separation factor (α) at 2.010. The other four tartrate compounds in the system gave much higher distribution ratios that are not suitable for CCC separation (Table 1).

Finally, the biphasic recognition chiral extraction was performed using the solvent system composed of *n*-hexane-MtBE-0.1 molL⁻¹ phosphate salt buffer solution with pH=2.68 (9:1:10, v/v/v) with the enantioselectivities for CHMA enantiomers at 2.01 which was slightly lower than that of the system 1, 2-dichloroethane-0.1molL⁻¹ phosphate salt buffer solution with pH=2.68 (1:1, v/v) with the enantioselectivities 2.49 [12]. The lower enantioselectivities in the above system may be chiefly due to the organic phase of *n*-hexane-MtBE (9 : 1, v/v) in which the tartrate could not have such higher chiral recognition as that in the system 1, 2-dichloroethane. But in the monophasic recognition chiral extraction system containing HP- β -CD in the aqueous, α for α -CHMA was 1.611 while in the monophasic recognition chiral extraction system containing (-)-tartrate [15-17], α is generally under 1.2, and therefore the biphasic recognition chiral extraction should achieve much better chiral separation than the monophasic recognition chiral extraction.

3.2. Effects of Various Parameters on Distribution Coefficient and Separation Factor

The effects of the concentration of (-)-2-ethylhexyl tartrate in the organic phase and HP- β -CD in the aqueous phase on distribution coefficient and separation factor were summarized in Figs. 2 and 3, respectively. With an increase of (-)-2-ethylhexyl tartrate content, the distribution coefficients for enantiomers were increased and the enantioselectivity reached the maximum when the concentration of tartrate was up to 0.3 mol L⁻¹. When the concentration of tartrate is further increased, the distribution coefficients increased continuously, while the enantioselectivity was decreased. On the other hand, with the increase of the concentration of HP- β -CD, the distribution coefficients for CHMA enantiomers decreased greatly and the enantioselectivity increased up to the concentration of HP- β -CD at 0.1 mol L⁻¹ and when the concentration of HP- β -CD was over 0.1 mol L⁻¹ the distribution coefficients and enantioselectivity began to decrease. Over all, the enantioselectivity of CHMA enantiomer reaches a maximum at the ratio of 3:1 in the molar concentrations of tartrate to HP- β -CD. Basically, the above results were consistent with that in the literature [12]. The fact that the enantioselectivity in the present study was not as high as that in the literature may be explained by the difference in the composition of the two-phase solvent system. Since the reported two-phase solvent system composed of 1, 2-dichloroethane-0.1molL⁻¹ phosphate salt buffer solution (1:1, v/v) was not applied in the CCC separation, the new solvent system *n*-hexane-MtBE-0.1 molL⁻¹ phosphate salt buffer solution with pH=2.68 (9:1:10,v/v/v) was employed,

Influence of pH was shown in Fig. 4. The distribution coefficients and enantioselectivity of CHMA enantiomers decrease as the pH of the aqueous phase is increased, probably because the ionic CHMA is formed with high pH in the aqueous phase. HP- β -CD mainly has chiral recognition ability and affinity for molecular CHMA, but not for ionic CHMA [12]. The concentration of complexes formed by (-)-2-ethylhexyl tartrate and enantiomers decreases as pH is increased. As a result D_R , D_S , and α were all remarkably decreased with the rise of the pH. Therefore, pH=2.68 was selected for the CCC separation.

The influence of temperature on the distribution behavior was investigated in the range of 2–45 °C. Table 2 shows that higher temperatures led to an increase in the distribution coefficient of (+)-CHMA and a slight decrease in the distribution coefficient of (-)-CHMA resulting in the decrease of the separation factor (α). The variations of $\ln k$ and $\ln \alpha$ versus $1/T$ in the range of 2-25 °C was shown in Fig. 5, where the results were fitting very well with the Van't Hoff model within the range of 2-25 °C, indicating that the complexes do not change in conformation and that enantioselective interactions remain unchanged.

3.3. Mechanism on Chiral Separation with Biphasic Recognition

The following is the glossary for the nomenclatures and symbols used in chiral CCC.

A₊: (+) isomer.

A_- : (–) isomer.

$[A_{\pm}]$: the concentration of both (+) and (–) isomers.

$[A_+]$ the concentration of (+) isomer.

$[A_-]$ is the concentration of (–) isomer.

CS: chiral selector, D-2-ethylhexyl tartrate or $_{\text{HF}}\beta\text{-CD}$.

$[\text{CS}]_{\text{ini}}$: the initial concentration of the chiral selector.

$[\text{CS}]_{\text{org}}$: actual concentration of CS in the organic phase (generally used as stationary phase).

$[\text{CS}]_{\text{aq}}$: actual concentration of CS in the aqueous phase (generally used as the mobile phase).

CSA: Chiral selector – enantiomer complex.

D ; the distribution ratio or the ratio of the total analytical concentration of a solute in one phase (regardless of its chemical form) to its total analytical concentration in the other phase.

K_D or D_o , :the partition ratio as the ratio of the concentration of a substance in a single definite form in one phase to that in the other phase at equilibrium. The distribution ratio governs the retention of a solute, while the partition ratio is useful for several analytical calculations.

$k_{f\pm}$: the complex formation constant of $[\text{CSA}_+]_{\text{org}}$ or $[\text{CSA}_-]_{\text{org}}$ in the case of 1:1 stoichiometry in the organic phase.

$k_{f\pm}'$: the complex formation constant of $[\text{CSA}_+]_{\text{aq}}$ or $[\text{CSA}_-]_{\text{aq}}$ in the case of 1:1 stoichiometry in the aqueous phase.

The quadratic scheme of the equilibrium between CS and $A_{\pm}$ in CCC originally used by Oliveros et al. [18] can be greatly simplified in the conventional chiral separation with monophasic recognition described by Ma et al. [3] based on the assumption that both CS and CSA remain only in the stationary (Fig. 6).

In this case (1:1 stoichiometry), the distribution ratio of $A_{\pm}$ is expressed as follows:

$$D_{\pm} = D_o \{1 + k_{f\pm} [\text{CS}]_{\text{org}}\} \quad (1)$$

And the separation factor, α , is expressed as (+ more retained than – enantiomer in the organic phase):

$$\alpha = D_+ / D_- = \frac{1 + k_{f+} [\text{CS}]_{\text{org}}}{1 + k_{f-} [\text{CS}]_{\text{org}}} \quad (2)$$

The above equation (2) indicates that the enantioseparation factor increases with the CS concentration and with the magnitude of the k_{f+}/k_{f-} ratio (monotonous increasing function).

Compared with the chiral CCC separation using monophasic recognition, the chiral CCC separation using biphasic recognition can be expressed by Fig. 7.

In this case (1:1 stoichiometry), the distribution ratio of $A_{\pm}$ is expressed as follows:

$$D_{\pm} = D_o \frac{1 + k_{f\pm} [\text{CS}]_{\text{org}}}{1 + k'_{f\pm} [\text{CS}]_{\text{aq}}} \quad (3)$$

And the separation factor, α , is expressed as:

$$\alpha = D_+ / D_- = \left\{ \frac{1 + k_{f+} [\text{CS}]_{\text{org}}}{1 + k_{f-} [\text{CS}]_{\text{org}}} \right\} \left\{ \frac{1 + k'_{f-} [\text{CS}]_{\text{aq}}}{1 + k'_{f+} [\text{CS}]_{\text{aq}}} \right\} \quad (4).$$

Equation (4) shows that the enantioseparation factor α increases with both of the hydrophilic CS concentration and the lipophilic CS concentration and with the magnitude of both of the k_{f+}/k_{f-} ratio in the organic phase and of the k'_{f-}/k'_{f+} ratio. Therefore, the value of enantioseparation factor, α , which is based on biphasic recognition, is generally larger than that based on monophasic recognition.

On the other hand, distribution ratio, separation factor and difference in free energy between the two diastereomeric complexes ($-\Delta(\Delta G)$) are important parameters to estimate the chiral CCC separation performance of CS, which can be calculated by the following formulas: $-\Delta(\Delta G) = RT \ln \alpha$, the driving forces $-\Delta(\Delta G)$ for the separation of CHMA are larger in the chiral separation by CCC based on biphasic recognition [12]. As a result, the enantioseparation factor α was improved greatly using biphasic recognition, which leads to stronger CCC separation ability than that with monophasic recognition.

3.4. Loading Limits for Chiral CCC Separation of α -CHMA Racemic Mixture

At a given initial CS concentrations, the loading limits (sample size) for the chiral CCC separation of α -CHMA was investigated to facilitate the preparative qualities of CCC and further scaling-up. To determine this, it is necessary to know the isotherms for (+) and (−) in the given biphasic system with a given initial CS concentrations. Most of the time, equilibrium such as that shown in Fig. 8 leads to Langmuirian isotherms:

$$[A_{\pm}]_{\text{org}} = \frac{a_{\pm} [A_{\pm}]_{\text{aq}}}{1 + b_{\pm} [A_{\pm}]_{\text{aq}}}$$

where $[A_{\pm}]_{\text{org}}$ indicates total analytical concentration of $A_{\pm}$ in the organic stationary phase, including free $[A_{\pm}]$ and complex $[CSA_{\pm}]_{\text{org}}$. $[A_{\pm}]_{\text{aq}}$ indicates total analytical concentration of $A_{\pm}$ in the aqueous mobile phase, including free $[A_{\pm}]$ and complex $[CSA_{\pm}]_{\text{aq}}$.

For an initial concentration $[A_{\pm}]^0$ in one volume of each phase, there is relationship for mass conservation:

$$[A_{\pm}]^0 = [A_{\pm}]_{\text{org}} + [A_{\pm}]_{\text{aq}}.$$

A series of batch experiments was performed with HPLC by varying the total concentrations of α -CHMA from 0.1 mmol L^{−1} to 50 mmol L^{−1} in the biphasic solvent system with lipophilic CS and hydrophilic CS. The results showed that equilibrium of α -CHMA in the biphasic system could be well described by Langmuirian isotherms. Fig. 8 shows Langmuirian isotherms and estimation of the operating conditions in the chiral CCC separation of α -CHMA.

During the chiral CCC separation with monophasic recognition, it was found that the best separation of a racemate will be achieved by applying a high CS concentration in the organic phase while adjusting the hydrophobicity of the solvent system so that the distribution coefficient of the racemate falls between 0.6 and 0.8 [3]. The separation factor increases with the hydrophobicity of the solvent system because the higher hydrophobicity of the solvent system decreases D_o and this in turn increases $[CS]_{org}$ in the stationary phase by shifting the balance of $[A+]_{org}+[CS]_{org}=[CSA+]_{org}$ toward the left. However, the separation of biphasic recognition chiral CCC depends on the cooperation of the CSs in both organic and aqueous phases. So the optimum distribution coefficient of the racemate by biphasic recognition chiral CCC separation is different from that of monophasic recognition chiral CCC, which might need further research to draw a conclusion about the optimum value of distribution coefficient in the biphasic recognition chiral CCC. Usually the higher hydrophobicity of a solvent system leads to the less solubility for a racemate. Much attention should be focused on the preparative quality of CCC which is easily scaled-up. The HPLC batch experiments for different concentrations of α -CHMA in the same biphasic solvent system showed that when the concentration of α -CHMA racemate rises to 12 mmol L^{-1} (6 mmol L^{-1} of each enantiomer) the enantioseparation factor began to decrease. So the maximum sample size for this separation was 12 mmol L^{-1} under the optimum conditions.

Before conducting the biphasic recognition chiral CCC separation of CHMA racemate, the retention of the stationary phase of the solvent system was measured and it was about 50%. Then the maximum sample size could be calculated. Taking the preparative column as an example, the retention volume of the organic stationary phase was 130 mL, and the loading limits for preparative separation was $12 \text{ mmol L}^{-1} \times 130 \text{ mL} \times 234.1 \text{ g mol}^{-1} = 365.2 \text{ g}$.

3.5. Chiral Separation of α -CHMA Racemic Mixtures by CCC

Enantioseparation of α -CHMA racemate was carried out using both analytical TBE-20A and preparative TBE-300A instruments. Fig. 9 shows the separations of α -CHMA enantiomers using the standard CCC technique. With a two-phase solvent system composed of *n*-hexane-MtBE-0.1 mol L^{-1} phosphate salt buffer solution with pH=2.68 (9:1:10, v/v/v), the separations were performed on an analytical scale (3.5-22.0 mg for racemic mixture). In each separation the sample solution was injected after hydrodynamic equilibrium was reached. Results showed that the resolution factor did not change in the sample size ranging from 3.5 to 22 mg, and the retention of stationary phase was 45.5% in all separations.

The above conditions for analytical separation were directly applied for the preparative separation for scaling up using the TBE-300A. apparatus Fig. 10 shows the CCC chromatogram for preparative enantioseparation of 365 mg of α -CHMA racemic mixtures. The retention for the stationary phase was 51.0% in this separation.

The racemate of CHMA and CCC fractions were analyzed by reverse HPLC with chiral mobile phase additives. Fig. 11 shows the HPLC chromatogram of CHMA enantiomer. The retention time of (+)- α -CHMA ($t=20.065 \text{ min}$) is less than that of (-)- α -CHMA ($t=22.185 \text{ min}$). HPLC results demonstrated that the purity of both of the ($\pm$)-CHMA enantiomer were over 99.5% and the enantiomeric excess (ee) of both of ($\pm$)- α -CHMA reached 100%. Therefore, the racemic mixture of α -CHMA could be completely separated by the biphasic recognition CCC technique and 365 mg of the sample was purified by the preparative CCC apparatus. Compared with the method of chiral extraction for resolution of α -CHMA racemate, in which the ee value for (+)- α -CHMA only reached 27.6%, the chiral CCC technique showed much advantages. Furthermore, this technique is easy to be scaled up.

Purification of the ($\pm$)-CHMA enantiomer from the CCC fractions were carried out by the silica gel column chromatography to remove the CSs, HP- β -CD and (-)-2-ethylhexyl tartrate.

Recovery of the ($\pm$)-CHMA enantiomer were both in the range of 85-88% with the purity of over 99.5%. Recovery of chiral selectors after CCC runs was not attempted

The optical activity of ($\pm$)- α -CHMA enantiomers from the chiral CCC fractions was determined by an Autopol I automatic polarimeter (Rudolph Research Analytical, USA). The optical rotation for the fraction from the first peak from the preparative chiral CCC separation was $[\alpha]_{\text{D}}^{15\circ}\text{C}(\text{methanol}) = +15.30$, and the optical rotation for the fraction from the second peak was: $[\alpha]_{\text{D}}^{15\circ}\text{C}(\text{methanol}) = -15.30$.

4. Conclusions

A novel chiral separation method for complete resolution of the racemic mixture ($\pm$)- α -CHMA enantiomers with preparative scale was established. Chiral CCC technique with biphasic recognition was performed by adding the lipophilic CS (–)-2-ethylhexyl tartrate to the organic stationary phase and the hydrophilic chiral selector HP- β -CD to the aqueous mobile phase. All the main factors that would influence the separation conditions were investigated. The two-phase solvent system *n*-hexane-MtBE-0.1 mol L^{–1} phosphate salt buffer solution with pH=2.68 (9:1:10, v/v/v) was used as the CCC solvent system with 0.3 mol L^{–1} (–)-2-ethylhexyl tartrate in the stationary phase and 0.1 mol L^{–1} HP- β -CD in the stationary phase. The separations were performed at temperature 8 °C and at pH=2.68 in the aqueous mobile phase. The maximum sample size for the preparative CCC apparatus with column volume 260 mL was about 365 mg in each separation. Under the above separation conditions, the purities of fractions from the preparative CCC separation were over 99.5% and ee reached 100% for the ($\pm$)-enantiomers. Recovery for the target compounds from the CCC fractions using the silica gel column chromatography reached 85-88%. The optical activity of both ($\pm$)-enantiomers were determined for the first time with the results $[\alpha]_{\text{D}}^{15\circ}\text{C}(\text{methanol}) = +15.30$ for fraction from the first peak and $[\alpha]_{\text{D}}^{15\circ}\text{C}(\text{methanol}) = -15.30$ for the fraction from the second peak in CCC separation.

Chiral separations are still challenges for CCC due to the difficulty of finding efficient chiral selectors in liquid phases. Although our studies only demonstrate chiral separations of ($\pm$)- α -CHMA by the biphasic recognition chiral CCC technique, we believe that the method may be extended to the separation of the other racemic mixtures by choosing an appropriate pair of hydrophilic and lipophilic chiral selectors which can cooperate each other to achieve efficient chiral separation..

Acknowledgments

This work was supported by Department of Education of Zhejiang Province of P. R. China (Y200803791).

References

- [1]. Buchholz K, Schulte B, Gmehling J, Martens J. Chem. Eng. Technol 1998;21:404.
- [2]. Ma Y, Ito Y, Foucault A. J. Chromatogr. A 1995;704:75. [PubMed: 7599747]
- [3]. Ma Y, Ito Y. Anal. Chem 1995;67:3069. [PubMed: 8779424]
- [4]. Perez, E.; Minguillon, C., editors. Counter-current chromatography in the separation of enantiomers. Chiral Separation Techniques. 3rd Edition. Vol. 11. Wiley-VCH Verlag GmbH & Co. KGaA; Weinheim, Germany: 2007. p. 369
- [5]. Foucault AP. J. Chromatogr. A 2001;906:365. [PubMed: 11215897]
- [6]. Perez E, Minguillon C. J. Sep. Sci 2006;29:1379. [PubMed: 16894782]
- [7]. Ai P, Liu JC, Zi M, Deng ZH, Yan ZH, Yuan LM. Chin. Chem. Lett 2006;17:787.
- [8]. Perez E, Santos MJ, Minguillon C. J. Chromatogr. A 2006;1107:165. [PubMed: 16412450]

- [9]. Delgado B, Perez E, Santano MC, Minguillon C. *J. Chromatogr. A* 2005;1092:36. [PubMed: 16188558]
- [10]. Kim E, Koo YM, Chung DS. *J. Chromatogr. A* 2004;1045:119. [PubMed: 15378886]
- [11]. Franco P, Blanc J, Oberleitner WR, Maier NM, Lindner W, Minguillon C. *Anal. Chem* 2002;74:4175. [PubMed: 12199590]
- [12]. Tang KW, Chen YY, Huang KL, Liu JJ. *Tetrahedron: Asymmetry* 2007;18:2399.
- [13]. Ito Y. *J. Chromatogr. A* 2005;1065:145. [PubMed: 15782961]
- [14]. Heldin E, Lindner KJ, Pettersson C, Lindner W, Rao R. *Chromatographia* 1991;32:407.
- [15]. Viegas RMC, Afonso CAM, Crespo JG, Coelho IM. *Sep. Purif. Technol* 2007;53:224.
- [16]. Tan B, Luo GS, Wang JD. *Sep. Purif. Technol* 2007;53:330.
- [17]. Prelog V, Kovakevi M, Egli M. *Angew. Chem* 1989;28:1147.
- [18]. Oliveros L, Puertolas PF, Minguillon C, Camacho-Frias E, Foucault A, Goffic FL. *J. Liq. Chromatogr* 1994;17:2301.

Fig. 1.
Chemical structure of (±)-α-cyclohexylmandelic acid

Figure 2.
Effect of the concentration of (-)-2-ethylhexyl tartrate on K and α for CHMA enantiomers.
Aqueous phase: [HP- β -CD]=0.1 mol L⁻¹, pH 2.68 and temperature 8 °C.

Figure 3. Effect of the concentration of HP- β -CD on K and α for CHMA enantiomers. Organic phase: $[(-)\text{-}2\text{-ethylhexyl tartrate}] = 0.3 \text{ mol L}^{-1}$, pH 2.68 and temperature 8°C .

Figure 4. Influence of pH on K and α for CHMA enantiomers. Organic phase: $[(-)-2\text{-ethylhexyl tartrate}] = 0.3 \text{ mol L}^{-1}$, Aqueous phase: $[\text{HP-}\beta\text{-CD}] = 0.1 \text{ mol L}^{-1}$, and temperature 8°C .

Figure 5.

Influence of temperature on D and α for CHMA enantiomers. Organic phase: [(-)-2-ethylhexyl tartrate]=0.3 mol L⁻¹, Aqueous phase: [HP- β -CD]=0.1 mol L⁻¹, pH=2.68

Fig. 6. Schematic diagram of chemodynamic equilibrium between the racemates ($A_{\pm}$) and chiral selector (CS) in the separation column based on monophasic recognition.

Fig. 7. Schematic diagram of chemodynamic equilibrium between the racemates ($A_{\pm}$) and chiral selector (CS) in the separation column based on biphasic recognition.

Fig. 8. Langmuirian isotherms and estimation of the operating conditions in chiral CCC separation of α -CHMA. Parameters for Langmuirian isotherms: $a_+=1.594$; $b_+=-0.0322$; $a_-=-3.215$; $b_-=-0.053$.

Fig. 9.

Separations of $(\pm)\text{-}\alpha\text{-CHMA}$ by the analytical chiral CCC technique. Experimental conditions: column volume 20 mL; solvent system: *n*-hexane: MtBE-0.1 mol L⁻¹ phosphate salt buffer solution with pH=2.68 (9:1:10, v/v/v), 0.3 mol L⁻¹ (-)-2-ethylhexyl tartrate in the organic phase and 0.1 mol L⁻¹ HP-β-CD in the aqueous phase. (a) 3.5 mg, (b) 7 mg, (c) 12 mg and (d) 22 mg of α-CHMA racemate dissolved in 1 mL of the organic phase, respectively; Flow rate: 0.5 mL/min in the head-to-tail elution mode; revolution: 1820 rpm; analysis of fractions: optical rotation and HPLC; stationary phase retention: 45.5% of the total column capacity. The retention times of (+)-CHMA was less than that of (-)-CHMA.

Fig. 10.

Separations of ($\pm$)- α -CHMA by preparative chiral CCC technique. Experimental conditions: column volume 260 mL; solvent system: *n*-hexane-MtBE-0.1 mol L⁻¹ phosphate salt buffer solution with pH=2.68 (9:1:10, v/v/v) containing 0.3 mol L⁻¹ (-)-2-ethylhexyl tartrate in the organic phase and 0.1 mol L⁻¹ HP- β -CD in the aqueous phase; sample: 365 mg of α -CHMA racemate dissolved in 20 mL of the organic phase; Flow rate: 2.0 mL/min in the head-to-tail elution mode; revolution: 850 rpm; analysis of fractions: optical rotation and HPLC; stationary phase retention : 51.0% of the total column capacity. The retention time of (+)-CHMA was less than that of (-)-CHMA.

Fig. 11.

Chromatogram of HPLC analyses of α -CHMA racemate and its preparative chiral CCC separation fractions from Fig. 10. (a) racemic mixture; (b): preparative chiral CCC fraction containing (+)- α -CHMA; (c): preparative chiral CCC fraction containing (-)- α -CHMA; HPLC conditions: column: YMC-Pack ODS-A, 5 μ m particle size of the packing material (250 mm $\times$ 6 mm I.D.); mobile phase: 0.075 molL⁻¹ KH₂PO₄ aqueous solution: alcohol: acetonitrile (65:20:15, v/v/v) containing 9.5 mmolL⁻¹ β -CD; flow rate: 0.6 mL/min; UV wavelength: 220 nm; column temperature: 30°C. The retention time of (+)-CHMA was less than that of (-)-CHMA.

Table 1

Distribution ratio (D) and separation factor (α) of different tartrate compounds in organic phase of the solvent system n -hexane-MtBE-0.1 molL⁻¹ phosphate salt buffer solution with pH=2.68 (9:1:10, v/v/v).

Aqueous phase: 0.1 molL⁻¹ HP- β -CD, organic phase: 0.3 molL⁻¹ tartrate, temperature: 8 °C, '–' insoluble

Tartrate	D_+	D_-	α
<i>n</i> -butyl tartrate	6.916	13.541	1.958
<i>isobutyl</i> tartrate	-	-	-
<i>n</i> -pentyl tartrate	3.215	6.124	1.904
<i>n</i> -hexyl tartrate	8.055	13.176	1.636
2-ethylhexyl tartrate	1.933	3.885	2.010
<i>n</i> -octyl tartrate	2.158	4.293	1.989
<i>n</i> -dodecyl tartrate	-	-	-

Table 2

Influence of the temperature on the enantioseparation of CHMA enantiomers

Temp. °C	K_S	K_R	α
2	1.929	4.030	2.089
8	1.933	3.885	2.010
15	1.946	3.699	1.901
20	1.951	3.559	1.824
25	1.970	3.413	1.733
35	2.184	3.812	1.745
45	2.479	3.885	1.572