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Enantioseparation of phenylsuccinic acid by high speed counter-current chromatography using hydroxypropyl-β-cyclodextrin as chiral selector

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ABSTRACT

High speed counter-current chromatography (HSCCC) was successfully applied to resolution of phenylsuccinic acid (PSA) with hydroxypropyl-β-cyclodextrin (HP-β-CD) as chiral selector (CS). The two-phase solvent system composed of *n*-hexane–methyl *tert*-butyl ether–0.1 mol L^{-1} phosphate buffer solution with pH = 2.51 (0.5:1.5:2, v/v/v) was selected. Influence factors involved in the chiral separation were investigated, including the concentration of chiral selector, pH value of the aqueous phase, the separation temperature, and the thermodynamic parameters of inclusion complex were calculated. The complex formation constants were determined using analytical instrument. Two HSCCC elution modes were studied and peak resolution equation was discussed. Under optimum separation conditions, 712 mg of PSA racemate was separated using preparative apparatus. The purities of both of the fractions including (+)-PSA and (−)-PSA from the preparative CCC separation were over 98.5% determined by HPLC and enantiomeric excess of (+)-PSA and (−)-PSA reached 97.6% and 98.6%, respectively. Recovery for the target compounds from the CCC fractions reached 80–82% yielding 285 mg of (+)-PSA and 292 mg of (−)-PSA.

1. Introduction

Among the various kinds of resolution methods, such as crystallization techniques, chromatographic techniques, enantioselective membranes and chiral extractions, development and application of chiral chromatographic methods have attracted intense interest during the past three decades. 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. However, HPLC was very expensive when it was used for chiral separation, especially for preparative-scale separations because the chiral selector is chemically bonded to a solid support that serves as a stationary phase, it requires series of time-consuming complicated processes. So preparative chiral HPLC was generally applied to resolution of enantiomers with high added values.

High speed counter-current chromatography (HSCCC) is a liquid–liquid chromatographic technique, with no solid support, in which the stationary and the mobile phases are constituted by two immiscible liquids or solutions. This method allows the separation of solutes in a two-phase solvent system subjected to a gravitational field, where the solutes that are to be separated are partitioned on the basis of their different affinity for one or the other phase [1]. This technique greatly increases the loading capacity of the stationary phase compared with HPLC and it is especially suited for preparative purposes [2]. However, the number of literatures about chiral HSCCC separation was much smaller than that of HPLC. The main reason for this phenomenon may be due to its relatively lower efficiency compared with HPLC. HSCCC need relatively greater separation factor ($\geq$1.4) to completely resolve two components. Another reason is the difficulty of finding suitable 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. Literatures about chiral separation using counter-current chromatography (CCC) and centrifugal partition chromatography (CPC) before 2001 had been reviewed [3] and totally 14 papers about chiral separation by CCC and CPC have been reported after 2001 [4–17]. So far the following nine chiral selectors had been successfully applied in chiral separation by CCC and CPC: cinchona alkaloid derivatives [4,5], *N*-dodecanoyl-L-proline derivatives [3,6], β-cyclodextrin derivatives [3,7–10], vancomycin [3], cellulose and amylose derivatives [11,12], (+)-(18-crown-6)-tetracarboxylic acid [13], tartaric acid derivatives [3,9,14], (*S*)-naproxen derivatives [15,16] and fluorinated chiral selectors [17].

Hydroxypropyl-β-cyclodextrin (HP-β-CD) derivatives could form inclusion complexes with various organic compounds in aqueous solution and they were frequently used as chiral selectors in HPLC [18]. In the present studies, HP-β-CD was used as chiral

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selector for HSCCC resolution of phenylsuccinic acid (PSA). PSA is an aromatic dicarboxylic acid used for the synthesis of *N*-methyl-α-phensuximide which is used as raw drug material.

2. Experimental

2.1. Apparatus

Two different models of HSCCC apparatus were used: TBE-20A analytical and TBE-300A preparative multilayer coil planet centrifuges (Shanghai Tauto Biotechnique, 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 TBE-300A preparative column consists of 1.6 mm ID PTFE tubing with a total capacity of 270 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 column coils 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. Both CCC centrifuges separation columns were installed in a vessel that maintains column temperature at 5 °C. 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 Biotechnique, 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 wavelength, and SEPU3000 workstation (Hangzhou Puhui Technology, Hangzhou, China) was employed to record the chromatogram.

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. The optical activity was determined by an automatic polarimeter Autopol I (Rudolph Research Analytical, USA).

2.2. Reagents

HP-β-CD was purchased from Xinda Fine Chemical & Co. Inc., Shandong, China. Phenylsuccinic acid (PSA) was purchased from Xiangfan Nuoer Chemical Co., Ltd, Hubei, China. All organic solvents used for HSCCC separation were of analytical grade. Acetonitrile (AcN) used for HPLC analysis was of chromatographic grade. Methyl *tert*-butyl ether (MtBE) was redistilled before use.

2.3. Liquid–liquid extraction experiments

To determine the distribution ratio of PSA racemate is essential for selection of the solvent system previous to the HSCCC study. The quantitative distribution of racemate in the biphasic solvent system was determined by means of liquid–liquid extraction experiments [11]. The organic/aqueous solvent systems were prepared in advance and allowed to equilibrate over 2 h. Two milliliters of the organic phase and two milliliters of the aqueous phase containing 0.05 mol L^{-1} of HP-β-CD and 1 mmol L^{-1} racemic PSA were added to 10 mL glass-stoppered tube. Then it was shaken vigorously for 10 min. The distribution of racemate was analyzed by HPLC. The solvent systems that gave the suitable distribution ratio (0.2–5) for (+)-PSA and (−)-PSA were considered for the development of the CCC separation.

2.4. Effects of influence factors on separation factor and distribution ratio

The following procedure was conducted to study the effects of influence factors on distribution ratio and enantioseparation factor. The aqueous phases were prepared by dissolving HP-β-CD and racemic PSA in a 0.1 mol L^{-1} phosphate buffer solution (pH = 2.51). The organic phase was composed of *n*-hexane–MtBE (0.5:1.5, 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 individual concentration of PSA enantiomers in the aqueous and organic phases was determined by HPLC.

2.5. Preparation of CCC solvent systems and sample solutions

Solvent systems consisting of *n*-hexane–MtBE–0.1 mol L^{-1} phosphate buffer solution with pH = 2.51 (0.5:1.5:2, 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 aqueous phase. The sample solutions were prepared as follows: 4 mg of PSA racemate was dissolved in 1 mL of the organic phase for analytical separations; 712 mg of PSA racemate was dissolved in 20 mL of the organic phase for preparative separations.

2.6. General CCC procedure

Both of analytical and preparative separations were initiated by filling the column with the stationary phase. The mobile phases were pumped into the column while the column was rotated at 1820 rpm for analytical separations and 850 rpm for preparative separations. Two elution modes including head-to-tail (aqueous phase as mobile phase) and tail-to-head (organic phase as mobile phase) modes were studied and compared. Each of the sample solution was injected after the hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the outlet.

2.7. Recovery of solutes from chiral CCC fractions in head-to-tail elution mode

A recovery method for PSA enantiomer was established when head-to-tail elution mode (aqueous phase as mobile phase) was used because solutes would be eluted with chiral selectors: the collected aqueous mobile phase fractions containing the separated enantiomers were acidified with a small volume of concentrated hydrochloric acid and extracted three times with MtBE. The combined organic layers were dried with anhydrous sodium 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:glacial acetic acid (10:1:0.05, v/v). The visual detections were done by concentrated sulfuric acid vapor. The experimental results showed that R_f value of (±)-enantiomer spots on the TLC was 0.34, R_f values of HP-β-CD was less than 0.05. The residue was further subjected to the silica gel column chromatography with isocratic elution (chloroform:methanol:glacial acetic acid, 10:1:0.05) to remove the small amount of HP-β-CD.

2.8. Analytical method

The quantification of PSA enantiomers was performed by HPLC using a UV detector set at 225 nm. The column was YMC-Pack ODS-A, with 5 μm particle size of the packing material, 250 mm × 4.6 mm I.D. (YMC Co., Ltd., Kyoto, Japan). The mobile phase was 10 mmol L^{-1} HP-β-CD aqueous solution:acetonitrile:trifluoroacetic acid (80:20:0.05, v/v/v) (pH = 2.5, adjusted with triethylamine) at a flow rate of 0.6 mL/min. The column temperature was 30 °C. The retention time of the (+)-PSA was less than that of the (−)-PSA.

3. Results and discussion

3.1. Selection of two-phase solvent systems

The major difficulty for successful enantioseparation of racemates by HSCCC is to find a suitable CS that is highly selective in the liquid phase as well as determination of suitable two-phase solvent system, which should not affect CS's selectivity and retains the capacity to elute chiral isomers of interest. Chiral selector used in HSCCC is always come from other separation techniques, such as HPLC, capillary electrophoresis, chiral extractions, etc. Several literatures showed that HP-β-CD could be used as CS in HPLC for resolution of PSA racemate [19,20]. In the literature [21] enantioselective separation of (±)-PSA by chiral extraction with HP-β-CD as CS in the aqueous phase was reported and high enantioseparation factor α = 1.93 was given for PSA enantiomers when the two-phase solvent system *n*-octanol:aqueous solution with pH = 2.5 containing 0.05 mol L^{-1} HP-β-CD was used. Since a fundamental feature of chiral extraction techniques (a separation mechanism governed by solution phenomena involving no solid support) is shared by HSCCC, we believe HP-β-CD could be applied for enantioseparation of PSA racemate by HSCCC if a suitable solvent system was found. The selection of this two-phase solvent system for the target compound(s) is the most important step in HSCCC where searching for a suitable two-phase solvent system may be estimated as 90% of the entire work in HSCCC [22,23]. The biphasic solvent system *n*-octanol:0.05 mol L^{-1} HP-β-CD aqueous solution with pH = 2.5 used in chiral extraction techniques could not be used in HSCCC separation because of its high degree of emulsification of the solvent system under 5 °C. So further efforts were needed to achieve the separation of enantiomers [4]. Liquid–liquid extraction experiments were necessary to find suitable solvent systems, which should satisfy the following requirements. First, HP-β-CD should be soluble only in aqueous phases. Second, the racemic mixtures should be easily soluble in both phases. Third, the optimum distribution ratio should be around 0.2–5. The following solvent systems were examined under 5 °C and distribution ratios of (±)-PSA were measured. All the aqueous phases used in the following systems was 0.1 mol L^{-1} phosphate buffer solution (pH = 2.51) added with 0.05 mol L^{-1} HP-β-CD. The two-phase solvent systems composed of *n*-hexane–aqueous phase (1:1, v/v), MtBE–aqueous phase (1:1, v/v), *n*-hexane–MtBE–aqueous phase (1.5:0.5:2, v/v/v), *n*-hexane–MtBE–aqueous phase (1:1:2, v/v/v), *n*-hexane–MtBE–aqueous phase (0.5:1.5:2, v/v/v), methyl isobutyl ketone–aqueous phase (1:1, v/v), *n*-hexane–methyl isobutyl ketone–aqueous phase (1.5:0.5:2, v/v/v) and *n*-hexane–methyl isobutyl ketone–aqueous phase (0.2:1.8:2, v/v/v) were tested. *n*-Hexane showed poor solubility for PSA, but MtBE and methyl isobutyl ketone showed good solubility for PSA. So the suitable values of distribution ratio of (±)-PSA for HSCCC separation could be obtained by changing the volume ratios of the above solvent systems. Totally three of the above systems were suitable for PSA resolution by HSCCC: the solvent system methyl isobutyl ketone–aqueous phase (1:1, v/v) gave D_+ = 1.55, D_- = 4.04, α = 2.61, *n*-hexane–methyl isobutyl ketone–aqueous phase (0.2:1.8:2, v/v/v) gave D_+ = 0.919, D_- = 2.23, α = 2.43 and the solvent system *n*-hexane–MtBE–aqueous phase (0.5:1.5:2, v/v/v) gave D_+ = 1.17, D_- = 2.43, α = 2.06. The two phase solvent system that containing methyl isobutyl ketone not only provided suitable distribution ratio and relatively larger enantioseparation factor for (±)-PSA, but also met requirements of solvent systems for HSCCC. So we had tried to use this system for resolution of PSA by analytical HSCCC with the molar ratio CS/racemate 25/1. Unfortunately, no successful enantioseparation was achieved due to unknown reasons. Under the above conditions, the solvent system *n*-hexane–MtBE–aqueous phase (0.5:1.5:2, v/v/v) provided good separation results and it was finally selected.

Table 1
Influence of the temperature on the enantioseparation of PSA enantiomers. Organic phase: *n*-hexane–MtBE (0.5:1.5, v/v), aqueous phase: [HP-β-CD] = 0.05 mol L^{-1}, pH = 2.51.

Temp. °C	D_-	D_+	α
5	2.527	1.208	2.092
10	2.241	1.216	1.842
15	2.104	1.200	1.753
20	2.082	1.195	1.741
25	1.814	1.170	1.551
30	1.850	1.167	1.585

3.2. Effects of influence factors on distribution ratio and separation factor

The effects of the concentration of HP-β-CD in aqueous phase on distribution ratio and enantioseparation factor were summarized in Fig. 1(a). With an increase of the concentration of HP-β-CD, the distribution ratio for PSA enantiomers decreased greatly and the enantioselectivity increased up to the concentration of HP-β-CD at 0.2 mol L^{-1}. When the concentration of HP-β-CD was over 0.2 mol L^{-1} the distribution ratios continue to decrease while enantioselectivity increased slightly. But large enantioseparation factor does not always lead to higher peak resolution if head-to-tail elution mode (aqueous phase as mobile phase) was used because increasing concentration of CS in the mobile phase generally results decreasing retention time of enantiomers. Series of separations were performed on an analytical HSCCC apparatus with HP-β-CD concentration 0.0 mol L^{-1}, 0.01 mol L^{-1}, 0.02 mol L^{-1}, 0.05 mol L^{-1}, 0.1 mol L^{-1}, 0.2 mol L^{-1} and 0.3 mol L^{-1}. The molar ratio CS/analyte was 25/1. Peak resolution was calculated from the chromatogram according the conventional formula:

$$R_s = \frac{2(V_{R2} - V_{R1})}{W_{b2} + W_{b1}} \quad (1)$$

where W_{b1} and W_{b2}, respectively, indicate the peak widths (4σ) of the first and the second peaks expressed in the same unit as V_{R1} and V_{R2}. Results showed that R_s value of PSA show convex curves with a maximum value at the concentration 0.05–0.1 mol L^{-1}, as shown in Fig. 1(a).

Influence of pH on the distribution ratio and separation factor was shown in Fig. 1(b). The distribution ratios and enantioselectivity of PSA enantiomers decreased as the pH of the aqueous phase is increased, because the ionic PSA is formed with high pH in the aqueous phase. HP-β-CD mainly has chiral recognition ability and affinity for molecular PSA, but not for ionic PSA. As a result D_-, D_+, and α were all remarkably decreased with the rise of the pH. Finally pH = 2.51 was selected for the HSCCC separation.

The influence of temperature on the distribution behavior was investigated in the range of 5–40 °C. Table 1 shows that higher temperatures led to a remarkable decrease in the distribution ratio

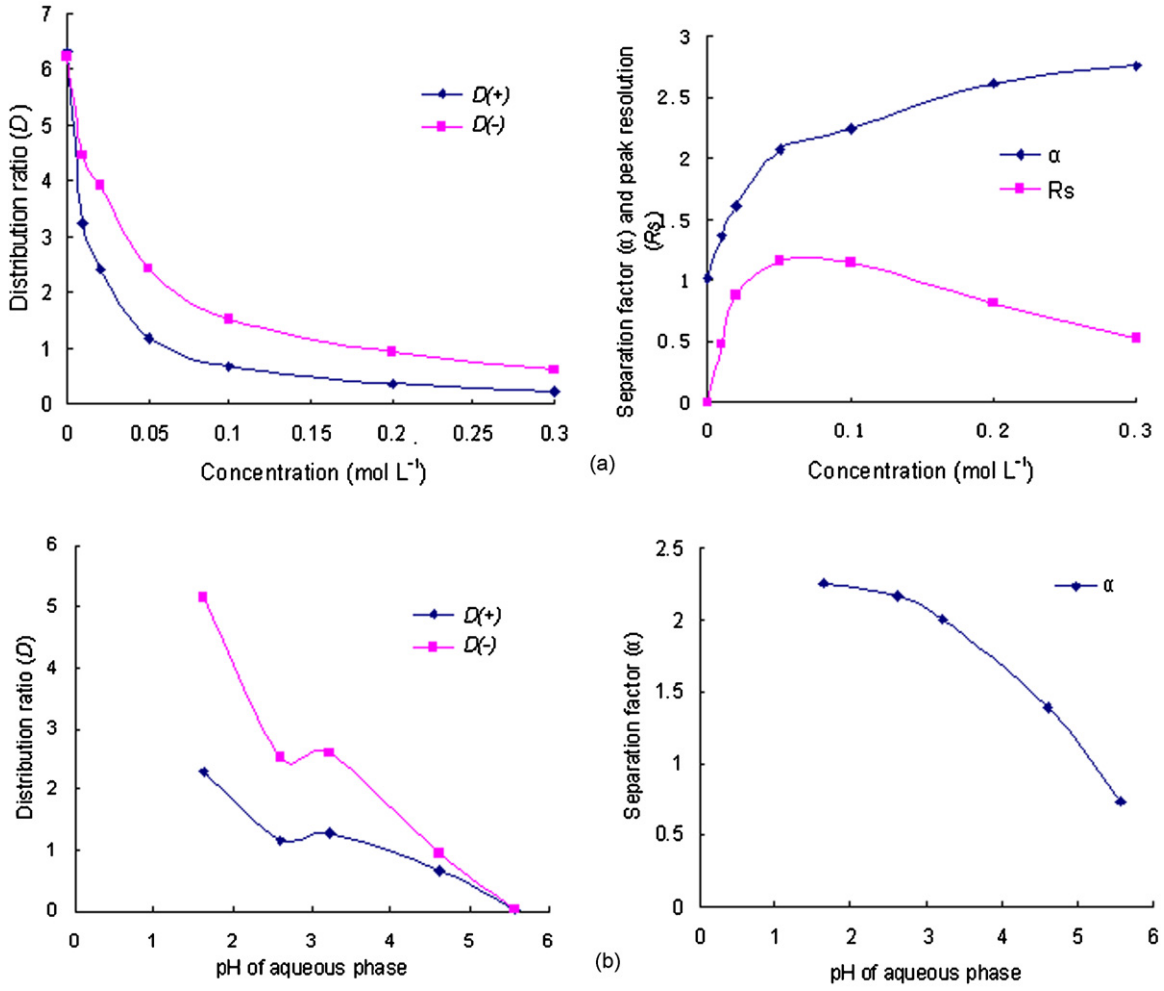

Fig. 1. (a) Effect of the concentration of HP-β-CD on D (distribution ratio) and α (enantioseparation factor) for PSA enantiomers—organic phase: n-hexane:MtBE = 0.5:1.5 (v/v), aqueous phase: 0.1 mol L^{-1} phosphate buffer solution with pH = 2.51, and temperature was 5 °C. (b) Influence of pH on D and α for PSA enantiomers—organic phase: n-hexane:MtBE = 0.5:1.5 (v/v), aqueous phase: [HP-β-CD] = 0.05 mol L^{-1}, and temperature 5 °C.

of (−)-PSA but no much difference was found as for the distribution ratio of (+)-PSA in the temperature range 5–30 °C, which led to a great decrease of the separation factor α. The variations of $\ln D$ and $\ln \alpha$ versus $1/T$ in the range of 5–30 °C were fitted very well with the Van't Hoff model within the range of 5–30 °C: $\ln \alpha = 844.99/T - 2.3324$, $R^2 = 0.9397$, indicating that the recognition and the chemical interactions between CS and enantiomers that lead to this recognition do not change within the studied range.

For enantiorecognition based on inclusion interactions of the host–guest complex, thermodynamic parameters of the complex formation should differ significantly. These parameters are accessible by the separation factor which is related to $\Delta_{\pm}(\Delta G)$, the difference in the molar Gibbs energy of the two enantiomers [24,25]. The enantioselectivity factor, α, is related to the difference between the free energies of association to the CS for the enantiomers of a racemic solute ($\Delta_{\pm}\Delta G$) by:

$$-\Delta_{\pm}(\Delta G) = RT \ln \frac{D_+}{D_-} = RT \ln \alpha \quad (2)$$

where the subscripts + and − refer to the two enantiomers, R is the gas constant ($R = 8.3143$ J K^{-1} mol^{-1}) and T the temperature in K.

The combination of above equation with the Gibbs–Helmholz relationship:

$$\Delta_{\pm}(\Delta G) = \Delta_{\pm}(\Delta H) - T\ \Delta_{\pm}(\Delta S) \quad (3)$$

Gives:

$$\ln \alpha = \frac{-\Delta_{\pm}(\Delta H)}{RT} + \frac{\Delta_{\pm}(\Delta S)}{R} \quad (4)$$

where $\Delta_{\pm}(\Delta H)$ and $\Delta_{\pm}(\Delta S)$ are the differences between the two enantiomers in enthalpy and entropy of complex formation, respectively. The differences between the two enantiomers in enthalpy and entropy of complex formation were $\Delta_{\pm}(\Delta H) = -7025.25$ J mol^{-1} and $\Delta_{\pm}(\Delta S) = -19.39$ J K^{-1} mol^{-1} which were calculated from the regression analysis and $\Delta_{\pm}(\Delta G) = -1634.83$ J mol^{-1} ($T = 278$ K) was obtained with the Gibbs–Helmholz equation. It has to be noted that $\Delta_{\pm}(\Delta H)$ is assumed to be independent of temperature in the range studied. A large negative term $T\Delta_{\pm}(\Delta S)$ represented an unfavorable influence of the entropy on $\Delta_{\pm}(\Delta G)$ and thus on the separation, but $\Delta_{\pm}(\Delta G)$ value was larger than that. Usually Gibbs energy of the inclusion complex formation is associated with a favorable proportion of enthalpy and an unfavorable entropy proportion. The obtained thermodynamic parameters demonstrated that the separation of PSA enantiomers was an enthalpy driven process, and the process of inclusion was exothermic process. $\Delta_{\pm}(\Delta G)$ is negative mainly owing to the contribution of $\Delta_{\pm}(\Delta H)$.

Table 2
The parameters of the distribution ratio D versus $[CS]_{ini}$.

$[CS]_{ini}$/mol L^{-1}	D_+	D_-	K_{D+}	K_{D-}	$(K_D)_\pm$	k'_{f+}/L mol^{-1}	k'_{f-}/L mol^{-1}
0	5.893	5.893					
0.02	3.200	4.296					
0.05	1.723	2.776	5.079	5.385	5.943	35.125	16.920
0.10	1.078	1.873					
0.20	0.644	1.259					

CS: chiral selector: HP-β-CD; D_+ and D_-: distribution ratio for enantiomers; K_D: partition coefficient of the enantiomer obtained by linear regression analysis; (K_D): partition coefficient of the enantiomer obtained by experiment with mobile phase free of CS; k'_f: formation constant of the CS–enantiomer complex.

3.3. Complex formation constant of CS-PSA by HSCCC with HP-β-CD as chiral selector

PSA enantiomers would mainly exist in the form of neutral molecule if pH ≤ 2.5 in the aqueous solution [21]. In the literature [26], a chromatographic method for measuring complex formation constant (k'_f) of the CS–enantiomer complexes by HSCCC was developed. This method could be applied in determination of formation constant of HP-β-CD–PSA enantiomers by changing the equation as the following Eq. (5) when HP-β-CD was added in the aqueous mobile phase (head-to-tail elution mode) and the CS would not partition to the organic stationary phase:

$$\frac{1}{D} = \frac{k'_f}{K_D}[CS]_{aq} + \frac{1}{K_D} \quad (5)$$

When the concentration of analytes is much smaller than that of the CS in the aqueous phase, $[CS]_{aq}$, Eq. (5) might be approximated as:

$$\frac{1}{D} = \frac{k'_f}{K_D}[CS]_{ini} + \frac{1}{K_D} \quad (6)$$

where $[CS]_{ini}$ was initial concentration of CS. The distribution ratio (D) of enantiomers can be calculated from HSCCC chromatogram according to the conventional equation:

$$D = \frac{V_R - V_m}{V_c - V_m} \quad (7)$$

The complex formation constant k'_f and partition coefficient K_D could be obtained by plotting $1/D$ versus CS concentration (slope = k'_f/K_D; intercept = $1/K_D$). If there is no change in the enantioseparation mechanism over the CS concentration range studied under constant pH value, the plot of the $1/D$ versus CS concentration yields a straight line. On the other hand, using the mobile phase free of CS under otherwise identical experimental conditions, the partition coefficient of the enantiomer is obtained according to the following equation:

$$K_D = \frac{V_o - V_m}{V_c - V_m} \quad (8)$$

where V_o is the retention volume of the enantiomer with the mobile phase free of CS. The K_D value obtained from Eq. (8) could further confirm the validity of the value of K_D calculated from Eq. (6).

Series of enantioseparation experiments of PSA racemate were carried out using head-to-tail elution mode (aqueous phase as mobile phase) by analytical TBE-20A instruments where 4 mg of PSA racemate was separated using various concentrations of the CS in the aqueous mobile phase. Fig. 2(a) shows the separations of PSA racemate using analytical HSCCC technique. With a two-phase solvent system composed of n-hexane–MtBE–0.1 mol L^{-1} phosphate salt buffer solution with pH = 2.51 (0.5:1.5:2, v/v/v), the following separations were performed on an analytical scale with HP-β-CD concentration 0.0 mol L^{-1}, 0.02 mol L^{-1}, 0.05 mol L^{-1}, 0.1 mol L^{-1}, 0.2 mol L^{-1} and 0.3 mol L^{-1}. The retention of the final separation was much lower (35%) than those of five other separations mainly due to its increasing viscosity of aqueous phase solution with high concentration of HP-β-CD. As expected, $1/D$ plotted against the initial CS concentration showed a straight line for each enantiomer with the HP-β-CD concentration ranged in 0.0–0.2 mol L^{-1}: $1/D_+ = 6.9161[CS]_{ini} + 0.1969$, $R^2 = 0.9960$ and $1/D_- = 3.1421[CS]_{ini} + 0.1857$, $R^2 = 0.9907$, which demonstrated no change happened in the enantioseparation mechanism over the CS

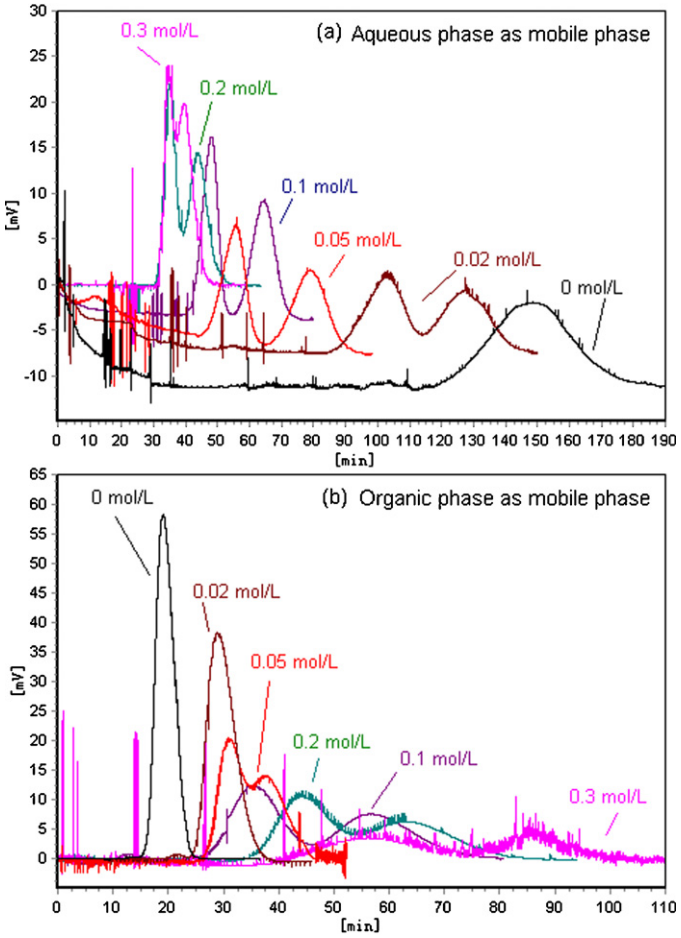

Fig. 2. Chromatogram of analytical chiral HSCCC for separation (±)-PSA with different concentration of HP-β-CD: 0–0.3 mol L^{-1}. Experimental conditions—solvent system: n-hexane:MtBE:0.1 mol L^{-1} phosphate salt buffer solution with pH = 2.51 (0.5:1.5:2, v/v/v); 4 mg of PSA racemate dissolved in 1 mL of the organic phase; flow rate: 0.5 mL/min; revolution: 1820 rpm; (a) head-to-tail elution mode, stationary phase: upper organic phase, mobile phase: lower aqueous phase; (b) tail-to-head elution mode, stationary phase: lower aqueous phase, mobile phase: upper organic phase. Stationary phase retention: (a) 0 mol L^{-1}: 55.5%; 0.02 mol L^{-1}: 55.0%; 0.05 mol L^{-1}: 55.0%; 0.1 mol L^{-1}: 52.5%; 0.2 mol L^{-1}: 45.5%; 0.3 mol L^{-1}: 35.0%. (b) 0 mol L^{-1}: 70.0%; 0.02 mol L^{-1}:52.5%; 0.05 mol L^{-1}: 50.0%; 0.1 mol L^{-1}: 49.5%; 0.2 mol L^{-1}: 45.5%; 0.3 mol L^{-1}: 27.5%.

concentration range (0–0.2 mol L^{-1}) studied with pH = 2.51 at 5 °C. The distribution ratio, partition coefficient and enantiomer CS complex formation constants calculated by the linear regression were listed in Table 2.

3.4. Selection of CCC elution mode and enantioseparation of PSA racemic mixtures by CCC

Chiral selectors were generally added in the stationary phase during the chiral CCC separations because CS could be retained in the stationary phase with no loss and analyte could be obtained directly. We first conducted our enantioseparation of PSA racemate by HSCCC with aqueous phase as stationary phase. Series of enantioseparation experiments of PSA racemate was carried out using the analytical TBE-20A instruments, in which organic phase was used as mobile phase. With a two-phase solvent system composed of *n*-hexane–MtBE–0.1 mol L^{-1} phosphate salt buffer solution with pH = 2.51 (0.5:1.5:2, v/v/v), the following separations were performed on an analytical scale with HP-β-CD concentration 0.0 mol L^{-1}, 0.02 mol L^{-1}, 0.05 mol L^{-1}, 0.1 mol L^{-1}, 0.2 mol L^{-1} and 0.3 mol L^{-1}. Fig. 2(b) shows HSCCC chromatogram for the separations of PSA racemic mixture. As the chromatogram shown, good peak resolution with suitable retention time was only obtained when HP-β-CD concentration was 0.1 mol L^{-1}. Increasing HP-β-CD concentration would lead to too long retention time with no better peak resolution. Preparative enantioseparation of PSA racemate was conducted with TBE-300A instruments. Fig. 3(a) shows typical HSCCC enantioseparation of PSA with aqueous phase containing CS as stationary phase. With the above two-phase solvent system added with 0.1 mol L^{-1} CS, 615 mg of PSA was injected. PSA racemic mixtures could not be completely separated with above tail-to-head elution mode (aqueous phase as stationary phase). However, as shown in Fig. 2(a), higher peak resolution with suitable retention time was observed if aqueous phase containing 0.05–0.1 mol L^{-1} HP-β-CD was used as the mobile phase. Preparative complete enantioseparation of PSA racemic mixture could be obtained with aqueous phase containing 0.05 mol L^{-1} chiral selectors as mobile phase under otherwise the same separation conditions (Fig. 3(b)). The separation was performed with 712 mg of PSA racemate. Elutes containing (+)-PSA and (−)-PSA were collected manually in the individual conical flask according to the HSCCC chromatogram. The shapes of peaks were strongly asymmetrical, indicating probably nonlinear isotherms existed. This is often encountered when complex formation is involved in one phase.

In order to theoretically explain why the elution mode with aqueous phase as mobile phase was better than the other elution mode, the exact resolution equation for counter-current chromatography was used. It was developed by Conway and Ito [27]:

$$R_s = \frac{1}{4}(\alpha - 1)\sqrt{N}\left(\frac{D_1}{D_1((\alpha+1)/2) + ((1-S_F)/S_F)}\right) \quad (9)$$

where N was the theoretical plates for HSCCC, D_1 was distribution ratio for the two enantiomers with $D_2 > D_1$ and S_F was the retention of stationary phase. Eq. (9) could be used to predict peak resolution for the enantiomers. N could be directly estimated from the HSCCC chromatogram using the following equation:

$$N = 16\left(\frac{V_R}{W_b}\right)^2 \quad (10)$$

The major factors contributing to the different peak resolution were separation factor α, theoretical plates N, distribution ratio D_1 and retention of stationary phase S_F, as shown in Eq. (9). As for the same two-phase solvent systems with different elution mode and the same HSCCC apparatus, the separation factor, theoretical plates and retention of stationary phase would be almost identical. So the value of D_1 plays a major role in affecting peak resolution. The larger of distribution ratio D_1, the better of peak resolution could be obtained. But too large distribution ratio would lead to too long retention time. The solvent system *n*-hexane–MtBE–0.1 mol L^{-1} phosphate buffer solution (pH = 2.51) added with 0.05 mol L^{-1} HP-β-CD (0.5:1.5:2, v/v/v) gave $D_+ = 1.17$, $D_- = 2.43$, $\alpha = 2.06$. When head-to-tail elution mode was used, $S_F = 55\%$, $D_1 = D_+$ and $N = 246$. The predicted R_s was 1.87 for the enantioseparation. While in tail-to-head elution mode, $S_F = 50\%$, $N = 240$, the separation factor was constant but $D_1 = 1/D_- = 0.412$. The predicted R_s would be only 1.04 for the enantioseparation. These predicted results were in agreement with the experimental results. A conclusion could be drawn that in head-to-tail elution mode (organic phase as stationary phase), better peak resolution was obtained because large D_1 value improved the chromatographic capacity factor. The aqueous solution with higher concentration of chiral selector, such as greater than 0.3 mol L^{-1} HP-β-CD, could also improved D_1 when tail-to-head elution mode (aqueous phase as stationary phase) was used. This might give better peak resolution as well. But the problem was the aqueous solution with high concentration of HP-β-CD would lead to serious emulsification due to its high viscosity. Therefore, the elution mode with aqueous phase as mobile phase was finally selected for present chiral HSCCC separation.

As for chiral HSCCC resolution of racemates, coelution of the two enantiomers may arise either by saturation of the chromatographic

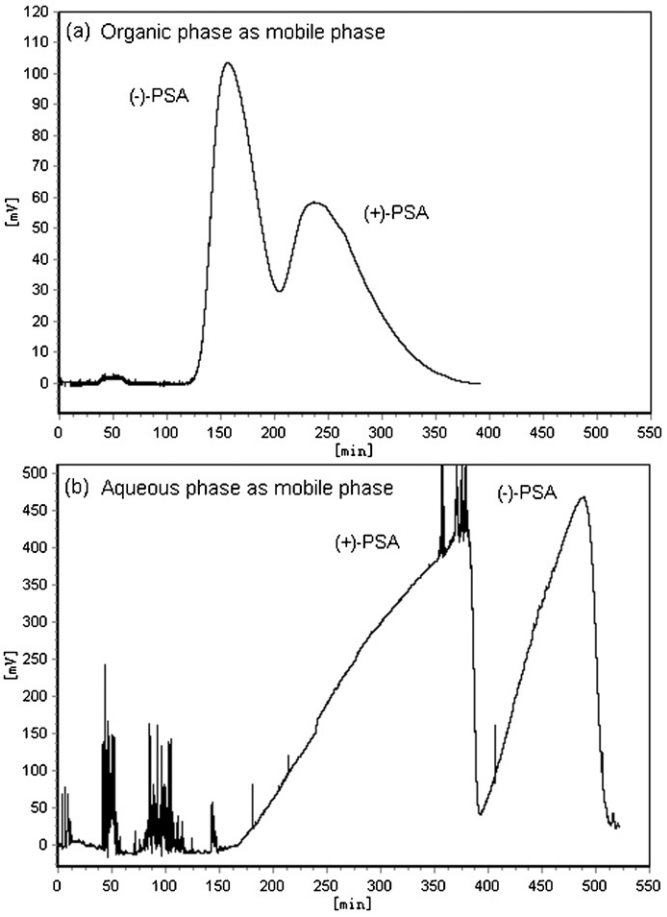

Fig. 3. Separations of (±)-PSA by preparative chiral CCC technique. Experimental conditions—solvent system: *n*-hexane–MtBE–0.1 mol L^{-1} phosphate salt buffer solution with pH = 2.51 (0.5:1.5:2, v/v/v) containing (a) 0.10 mol L^{-1} HP-β-CD and (b) 0.05 mol L^{-1} HP-β-CD in the aqueous phase; sample: (a) 615 mg and (b) 712 mg of PSA racemate dissolved in 20 mL of the organic phase; flow rate: 2.0 mL/min in the (a) tail-to-head elution mode organic phase as mobile phase and (b) head-to-tail elution mode aqueous phase as mobile phase; revolution: 850 rpm; stationary phase retention: (a) 91.5% and (b) 62.9% of the total column capacity.

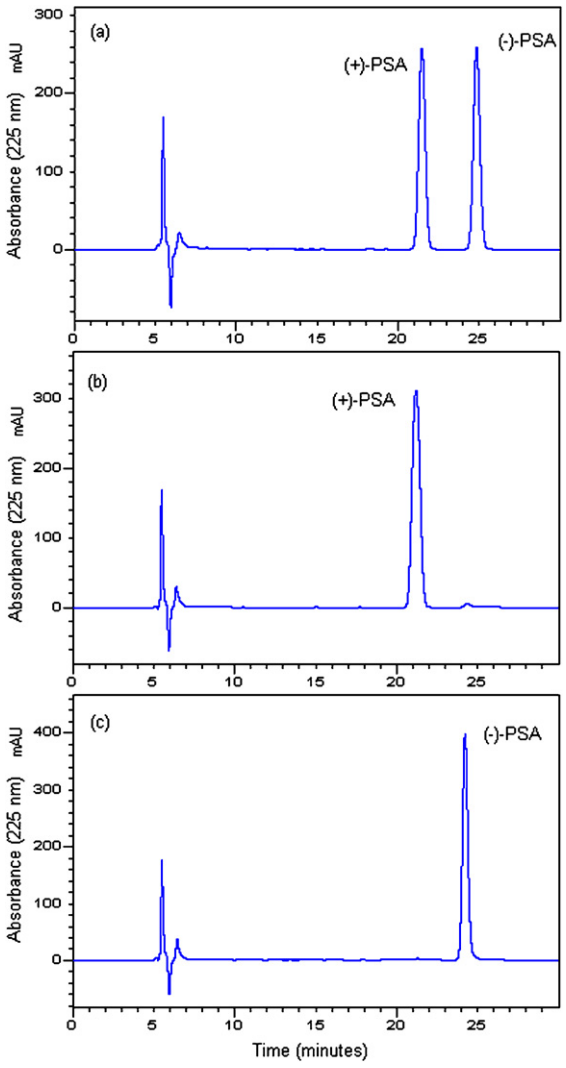

Fig. 4. Chromatogram of HPLC analyses of PSA racemate and its preparative chiral CCC separation fractions from Fig. 3(b): (a) racemic mixture; (b) preparative chiral CCC fraction containing (+)-PSA; (c) preparative chiral CCC fraction containing (−)-PSA. HPLC conditions: see Section 2.8.

system or as a result of the low enantioselectivity in the conditions studied [11]. A maximum molar ratio CS/analyte (1:1) was determined to the limit capacity in chiral CCC separation, in which CSs forms 1:1 complexes with enantiomers and showing high enantioselectivity for the analyte studied. Solubility of the analyte in the two phase solvent system used could also limit the concentration of the injected sample. The recommended sample volume in the standard separation using the semipreparative column of the commercial HSCCC unit with partition efficiency of about 600–800 TPs may be less than 5% of the total column capacity [22]. So the maximum amount of sample that can be dissolved in the organic phase (20 ml for the preparative apparatus) was investigated with the above influence factors. The results showed that the maximum amount of sample that could be dissolved in the organic stationary phase was 35.6 mg/mL (183.3 mmol L^{-1}) under 5 °C. As for the preparative separation of 712 mg racemic PSA with aqueous phase as mobile phase, the molar ratio HP-β-CD/analyte would be in the order of 2/1, which was adequate to avoid saturation of the chromatographic system. One of the beauties of CCC is that suspensions can be injected due to the absence of solid support, which could further increase injected sample size. Further increase in sample size was not investigated since the present molar ratio CS/analyte was near saturation of the chromatographic system.

The racemate of PSA and CCC fractions were analyzed by reverse HPLC (Fig. 4). The retention time of (+)-PSA (t = 21.479 min) is less than that of (−)-PSA (t = 24.852 min). HPLC results demonstrated that the purity of both of the (±)-PSA enantiomer was over 98.5% and the enantiomeric excess (ee) of (+)-PSA and (−)-PSA reached 97.6% and 98.6%, respectively. Therefore, PSA racemic mixture could be completely separated by the HSCCC using HP-β-CD as chiral selector. 712 mg of the racemate was separated by the preparative HSCCC apparatus.

Purification of the (±)-PSA enantiomer from HSCCC fractions were carried out by the silica gel column chromatography to remove the small amount of HP-β-CD. Recovery of both of the (±)-PSA enantiomer was in the range of 80–82% with the purity of over 98.5% and 285 mg of (+)-enantiomer and 292 mg of (−)-enantiomer were obtained from the preparative separation. Recovery of CS after CCC runs was not investigated. The optical rotation determined by the automatic polarimeter for the fraction with the less retention time in Fig. 3(b) was $[\alpha]_D^{30\,^\circ C}$(acetone) = +152, and the optical rotation for the fraction with the more retention time was $[\alpha]_D^{30\,^\circ C}$(acetone) = −160, which were in agreement with those optical rotation values reported in the literature [28].

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