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Enantiomeric separation of (*R*, *S*)-naproxen by recycling high speed counter-current chromatography with hydroxypropyl-β-cyclodextrin as chiral selector

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ABSTRACT

Recycling high speed counter-current chromatography (HSCCC) was successfully applied to resolution of (*R*, *S*)-naproxen (NAP) using hydroxypropyl-β-cyclodextrin (HP-β-CD) as chiral selector. The two-phase solvent system composed of *n*-hexane–ethyl acetate–0.1 mol L^{-1} phosphate buffer solution with pH = 2.67 (8:2:10, v/v/v) was selected. Influence factors for the chiral separation process were investigated, including concentration of HP-β-CD, equilibrium temperature and pH of aqueous phase. Suitable elution mode was selected for HSCCC enantioseparation of (*R*, *S*)-NAP. Under optimum separation conditions, 29 mg of (*R*, *S*)-NAP was separated using preparative recycling HSCCC with the molar ratio HP-β-CD/NAP racemate 83:1. Technical details for recycling elution mode were discussed as for chiral HSCCC separation. The purities of both (*S*)-NAP and (*R*)-NAP were over 99.5% as determined by HPLC. Enantiomeric excess of (*S*)-NAP and (*R*)-NAP reached 99.4%. Recovery for NAP enantiomers from HSCCC fractions was 82–89%, yielding 13 mg of (*S*)-NAP and 12 mg of (*R*)-NAP.

1. Introduction

2-Arylpropionic acid (2-APA) nonsteroidal anti-inflammatory drugs (NSAIDs) represent an important group of pharmaceutical compounds and exhibit optical activity characterized by a chiral carbon atom near the carboxylic acid group, such as naproxen, ibuprofen, ketoprofen, flurbiprofen, indoprofen, and carprofen. It is known that individual enantiomers of some 2-APA NSAIDs have different therapeutic activities [1]. They have their pharmacological activity mainly on the (*S*)-enantiomer [2]. (*S*)-naproxen (NAP), also named (*S*)-(+)-2-(6-methoxy-2-naphthyl) propionic acid, is a widely used NSAID in the treatment of rheumatoid arthritis [3]. (*S*)-naproxen showed 28 times higher activity than the (*R*)-isomer. The production of optically pure naproxen has been a hot subject, and some research groups have reported the enzymatic resolution [4] and kinetic resolution [5] of (*S*)-naproxen. High-performance liquid chromatography (HPLC) has been most frequently used for enantiomeric analysis for NAP [6–10].

High speed counter-current chromatography (HSCCC) belongs to liquid–liquid partition chromatographic technique. HSCCC use no solid support for the stationary phase and it allows preparative separation of solutes in a two-phase solvent system based on different affinities for one or the other phase. 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. Generally, HSCCC owns not more than 1000 theoretical plates during the separation. HSCCC need relatively greater separation factor to completely resolve two components. Another important reason is the difficulty of finding good chiral selectors that are highly selective in the liquid phase of the two-phase solvent system that does not affect their selectivity. As for the first reason, recycling elution mode could make up its deficiency to some extent and solutes with quite low separation factor could also be completely separated by this elution mode. Recycling elution mode was generally used in preparative HPLC separation to improve resolution factor. So far only two literatures [11,12] were available for using this technique in HSCCC separation. Two natural components with quite low separation factor were separated using recycling HSCCC. As a matter of fact, chances for low enantioseparation factor during chiral separation would be higher than that in the non-chiral separations. So recycling elution mode would be an ideal choice for chiral HSCCC separation. This methodology requires prolonged preparation, but the solvent consumption and the volume separated remain the same. This elution mode is easy to conduct and chiral selector could be either added in the stationary phase or in the mobile phase with no loss during the

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recycling elution. Most important of all, resolution factor for target enantiomers could be remarkably improved through this simple method.

Chiral separation using counter-current chromatography (CCC) and centrifugal partition chromatography (CPC) has been reviewed in the literature [13]. So far the following nine chiral selectors had been successfully applied in chiral separation by CCC and CPC: cinchona alkaloid derivatives [14,15], *N*-dodecanoyl-L-proline derivatives [16,17], β-cyclodextrin derivatives [18–21], vancomycin [22], cellulose and amylose derivatives [23,24], (+)-(18-crown-6)-tetracarboxylic acid [25], tartaric acid derivatives [20,26,27], (*S*)-naproxen derivatives [28,29] and fluorinated chiral selectors [30]. In the present study, hydroxypropyl-β-cyclodextrin (HP-β-CD) was used as chiral selector for HSCCC separation of (*R*, *S*)-naproxen using recycling elution mode.

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 analytical centrifuge, and from 0 to 1000 rpm for the preparative centrifuge where the optimum speed of 1820 rpm was used for the analytical columns and 850 rpm for preparative columns. Both CCC centrifuge separation columns were installed in a vessel that maintains column temperature at 5 °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 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 SEPU3000 workstation (Hangzhou Puhui Technology, Hangzhou, China) was employed to record the chromatogram. The recycling HSCCC separation was carried out on TBE-300A system with a column switching valve.

The high-performance liquid chromatography (HPLC) used was a CLASS-VP Ver.6.1 system (Shimadzu, Japan) comprised 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 of (*R*, *S*)-NAP enantiomers from the chiral CCC fractions 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. Naproxen racemate was purchased from J&K chemical Scientific Co., Ltd, Shanghai, China. All organic solvents used for HSCCC separation were of analytical grade. Methanol used for HPLC analysis was of chromatographic grade.

2.3. Liquid–liquid extraction experiments

To determine the distribution ratio of NAP is essential for the selection of the two-phase solvent system previous to HSCCC study. Distribution ratios are defined as follows: distribution ratios for NAP enantiomers were calculated by the concentration of NAP enantiomer in the organic phase divided by the concentration of NAP enantiomer in aqueous phase. The quantitative distribution of racemate in the biphasic solvent system was determined by means of liquid–liquid extraction experiments under 5 °C [23]. The aqueous solution was 0.1 mol L^{-1} phosphate buffer solution with pH = 2.67 containing 1 mmol L^{-1} NAP racemate and 0.1 mol L^{-1} chiral selector HP-β-CD. 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 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 for (*S*)-enantiomer and (*R*)-enantiomer were considered for the development of CCC separation. After the two-phase solvent system was determined, influence factors including concentration of HP-β-CD, equilibrium temperature and pH of buffer solution were further investigated with liquid–liquid extraction experiments. The organic phase was composed of *n*-hexane–ethyl acetate (8:2, v/v). Concentration of NAP racemate was 1 mmol L^{-1}. During the liquid–liquid extraction experiments for the investigation of pH and temperature the molar ratio of HP-β-CD/NAP racemate was 100:1.

2.4. Preparation of CCC solvent systems and sample solutions

Solvent systems consisting of *n*-hexane–ethyl acetate–0.1 mol L^{-1} phosphate buffer solution with pH = 2.67 containing 0.1 mol L^{-1} HP-β-CD (8:2:10, v/v/v) was used. The aqueous phase containing chiral selector used for recycling HSCCC operation need to be filtrated by HPLC water millipore filter. Then the solvent mixture was thoroughly equilibrated in a separatory funnel, and the two phases were separated and degassed by ultrasound for 30 min shortly before use. The sample solutions were prepared as follows: 1.5 mg of NAP racemate was dissolved in 1 mL of the organic phase for analytical separations and 29–30 mg of NAP dissolved in 20 mL of the organic phase for preparative separations.

2.5. HSCCC separation procedure

Conventional HSCCC: 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. Each of the sample solution was injected after the hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet. The effluent from the outlet of the column was continuously monitored at 280 nm. The separation temperature was controlled at 5 °C.

Recycling HSCCC: The difference between the conventional HSCCC and the recycling HSCCC is that the latter iteratively injects the effluent by channeling the outlet of the detector to the inlet of the pump. The whole procedure was carried out in the following three steps: step 1: the separation column was entirely filled with the upper phase of the solvent system. Then the coil column was rotated 850 rpm, while the lower phase was pumped into the column at 2.0 mL min^{-1}. After the mobile phase front emerged and

hydrodynamic equilibrium was established in the column, 20 mL of sample solution containing 29 mg of racemate was injected through the injector. Step 2: after the sample injection and immediately before the first cycle elution (retention time was 80–90 min), the switching valve was turned to form a recycling tube. Step 3: When the target racemate was completely separated after several CCC recycles, recycling elution mode was stopped to release the enantiomers. Each peak fraction was manually collected according to the chromatogram.

2.6. Recovery of solutes from chiral CCC fractions

A recovery method for NAP 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 HSCCC fractions containing the separated enantiomers were acidified with a small volume of concentrated hydrochloric acid and extracted three times with ethyl acetate. 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 (98:2, v/v). The experimental results showed that R_f value of (±)-enantiomer spots on the TLC was 0.48, R_f values of HP-β-CD was less than 0.05. In order to purify the NAP enantiomers, the residue was further subjected to the silica gel column chromatography with isocratic elution (chloroform:methanol = 98:2) to remove the small amount of HP-β-CD.

2.7. Analytical method

The quantification of NAP enantiomers was performed by HPLC using a UV detector set at 273 nm. The column was YMC-Pack ODS-A, with 5 μm particle size of the packing material, 150 mm × 4.6 mm I.D. (YMC Co., Ltd., Kyoto, Japan). The mobile phase was 25 mmol L^{-1} HP-β-CD aqueous solution: methanol:glacial acetic acid (60:40:0.25, v/v/v) (pH = 3.5, adjusted with triethylamine) at a flow rate of 0.7 mL min^{-1}.

3. Results and discussion

3.1. Selection of two-phase solvent systems

Several literatures have reported HPLC analysis of (*R*, *S*)-NSAIDs using HP-β-CD as chiral selector [6–10] and HPLC results showed that high peak resolution was obtained for (*R*, *S*)-naproxen and (*R*, *S*)-carprofen. As for chiral HSCCC separation, the most important step for successful enantioseparation is to find a suitable two-phase solvent system in which chiral selector is highly selective in the liquid phase. A suitable two-phase solvent system requires the following considerations: (1) retention of the stationary phase should be satisfactory; (2) the settling time of the solvent system should be short (i.e. <30 s); and (3) the partition coefficient of the target compound should fall within a suitable range (i.e. usually between 0.2 and 5) and (4) the chiral selector should be soluble only in one phase while the racemic mixtures should be easily soluble in both phases. Several solvent systems were examined and distribution ratios of (*R*, *S*)-NAP were measured under 5 °C (Table 1). (*R*, *S*)-NAP showed poor solubility in the aqueous solution without HP-β-CD. (*R*, *S*)-NAP could be soluble in the aqueous solution due to complex formation between NAP and HP-β-CD. As for the selection of optimum solvent system, 0.1 mol L^{-1} HP-β-CD was added in the aqueous solution. As shown in Table 1, suitable distribution ratio for NAP enantiomers could be obtained with the four solvent systems: *n*-hexane:methyl *tert*-butyl ether:aqueous solution (8:2:10), *n*-hexane:methyl *iso*-butyl ketone:aqueous solution (8:2:10), *n*-hexane:ethyl acetate:aqueous solution (8:2:10) and *n*-hexane:ethyl acetate:methanol:aqueous solution (1:1:1:1). However, the solvent system *n*-hexane:ethyl acetate:methanol:aqueous solution (1:1:1:1) gave the lowest enantioseparation factor (α = 1.011). Obviously, the solvent systems containing methanol lead to no enantio-recognition for chiral selector. In other words, the organic solvent methanol dissolved in the aqueous solution would lead to great decrease of separation factor. So totally three solvent systems were selected: *n*-hexane:methyl *tert*-butyl ether:aqueous solution (8:2:10), *n*-hexane:methyl *iso*-butyl ketone:aqueous solution (8:2:10), *n*-hexane:ethyl acetate:aqueous solution (8:2:10). They were further tested on the analytical HSCCC apparatus for the resolution of small amount of NAP racemate. Analytical HSCCC separation results showed that as for peak resolution, no much difference were observed for the above three systems. The solvent system *n*-hexane:ethyl acetate:aqueous solution (7:3:10) lead to too long retention time for NAP enantiomers when preparative HSCCC was conducted. Finally, the solvent system *n*-hexane:ethyl acetate:aqueous solution (8:2:10) was found to be suitable for recycling HSCCC enantioseparation of NAP.

The other 2-APA NSAIDs including carprofen, ibuprofen, flurbiprofen, ketoprofen and suprofen were also tested with the above selected solvent systems. Suitable distribution ratios were obtained for those racemates but all enantioseparation factor values were less than 1.15, which were too small to be separated by HSCCC and no successful separation were achieved.

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

Influence of pH value, temperature and concentration of HP-β-CD on distribution ratio and enantioseparation factor of NAP were investigated.

Naproxen is a weak acid compound (pKa = 4.2), whose solubility is strongly influenced by pH variations [31]. The distribution ratios and enantioselectivity of NAP enantiomers decreased as the pH of the aqueous phase was increased (Fig. 1) because the ionic NAP is formed with high pH in the aqueous phase. All the racemic NAP would be in the basic aqueous solution, either free or associated to the chiral selector. In such conditions the difference in association for the two enantiomers could not be observed. As a result D_R, D_S and α were all remarkably decreased with the rise of the pH. pH = 2.67 was selected.

The effects of concentration of HP-β-CD in aqueous phase on distribution ratio and enantioseparation factor were summarized in Fig. 2. With an increase of the concentration of HP-β-CD, the distribution ratio for NAP enantiomers decreased greatly and the enantioselectivity increased up to the concentration of HP-β-CD at 0.02 mol L^{-1}. When the concentration of HP-β-CD was over 0.1 mol L^{-1}, the distribution ratios slightly increased but enantioselectivity decreased greatly. D_S = 70.3 and D_R = 70.4 when the concentration of HP-β-CD was 0.0 mol L^{-1} in the aqueous phase. On the other hand, low solubility of naproxen was found in aqueous phase added with less than 0.1 mol L^{-1} HP-β-CD. The concentration of HP-β-CD 0.1 mol L^{-1} was selected as far as the solubility for NAP in the solvent system was considered.

The influence of temperature on the distribution behavior was investigated in the range of 5–50 °C. Table 2 shows that higher temperatures led to an increase in the distribution ratio of NAP enantiomers in the temperature range 5–40 °C while the separation factor α decreased greatly. The temperature 5 °C was selected for HSCCC separation. 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. The regression equation were as follows: $\ln \alpha = 571.39/T - 1.7046$, $R^2 = 0.9853$, $\ln D_S = -1166.3/T + 3.2564$, $R^2 = 0.9762$, $\ln D_R = -594.93/T + 1.5517$,

Table 1
The *D* (distribution ratio) and enantioseparation factor values of (*R*, *S*)-naproxen in different solvent systems.

Solvent system	D_S	D_R	α
Methyl *tert*-butyl ether:aqueous solution (1:1)	-	-	
Ethyl acetate:aqueous solution (1:1)	-	-	
Methyl *iso*-butyl ketone:aqueous solution (1:1)	-	-	
n-Hexane:aqueous solution (1:1)	--	--	
n-Hexane:methyl *tert*-butyl ether:aqueous solution (9:1:10)	0.22	0.31	1.41
n-Hexane:methyl *tert*-butyl ether:aqueous solution (8.5:1.5:10)	0.36	0.54	1.50
n-Hexane:methyl *tert*-butyl ether:aqueous solution (8:2:10)	1.12	1.62	1.45
n-Hexane:methyl *iso*-butyl ketone:aqueous solution (8:2:10)	0.99	1.45	1.46
n-Hexane:ethyl acetate:aqueous solution (8:2:10)	0.46	0.69	1.50
n-Hexane:ethyl acetate:aqueous solution (7:3:10)	1.28	1.80	1.41
n-Hexane:ethyl acetate:methanol:aqueous solution (3:5:3:5)	8.69	8.95	1.03
n-Hexane:ethyl acetate:methanol:aqueous solution (1:1:1:1)	1.91	1.93	1.01
Ethyl acetate:methanol:aqueous solution (10:1:10)	42.26	54.32	1.29

All the aqueous phases used in the above systems was 0.1 mol L^{-1} phosphate buffer solution (pH = 2.67) added with 0.1 mol L^{-1} HP-β-CD and 1.0 mmol L^{-1} naproxen racemate. Equilibration temperature: 5 °C; '-' and '--': distribution ratio was too large and too small, respectively.

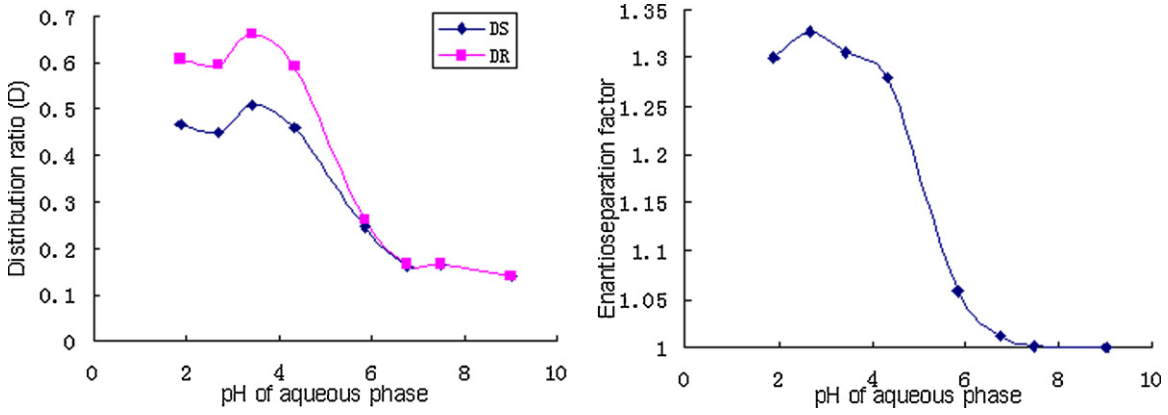

Fig. 1. Influence of pH on *D* (distribution ratio) and α (enantioseparation factor) for NAP enantiomers. Organic phase: *n*-hexane:ethyl acetate = 8:2 (v/v) containing 1 mmol L^{-1} NAP racemate, aqueous phase: [HP-β-CD] = 0.1 mol L^{-1}, the molar ratio of CS/racemate was 100:1, and temperature 5 °C.

R^2 = 0.9295, which indicated that the recognition and the chemical interactions between CS and enantiomers that lead to this recognition do not change within the studied range.

3.3. Selection of CCC elution mode and enantioseparation of NAP racemic mixtures by HSCCC

Two elution modes could be applied during HSCCC separation: head-to-tail elution mode and tail-to-head elution mode. As for enantioseparation of racemic mixtures by HSCCC, chiral selector was generally added in the stationary phase because the chiral selectors could be retained with no loss during the whole process and analyte could be obtained directly. Chiral selector added in the mobile phase need further purification step for analyte. So we first conducted our enantioseparation of NAP racemate by HSCCC with aqueous phase as stationary phase. Series of enantioseparation experiments of 1.5 mg of NAP racemate was carried out using the analytical TBE-20A instruments, in which tail-to-head elution mode was used. Retention of aqueous stationary phase was 75%. The molar ratio of HP-

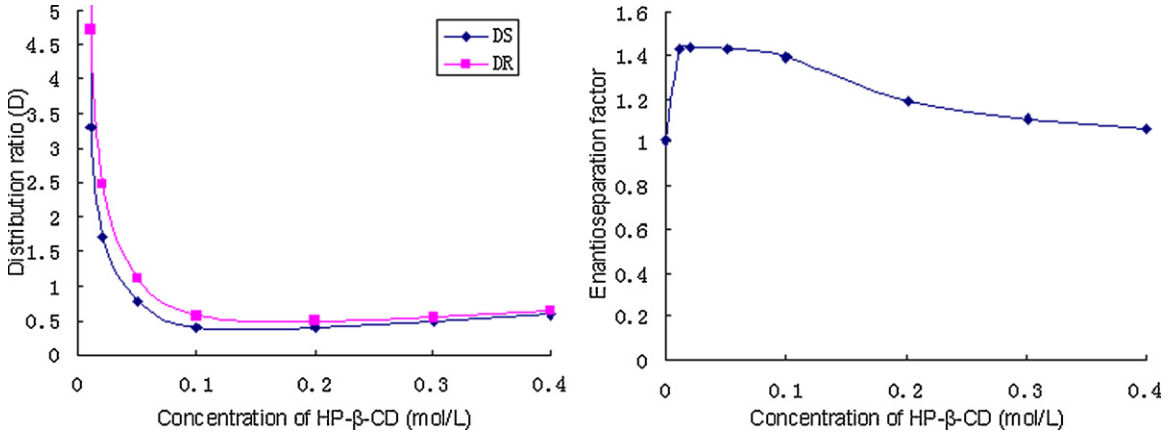

Fig. 2. Effect of concentration of HP-β-CD on *D* (distribution ratio) and α (enantioseparation factor) for NAP enantiomers. D_S = 70.3, D_R = 70.4 and α = 1.0 when the concentration of HP-β-CD was 0.0 mol L^{-1} in the aqueous phase and this is not shown in the figure. Organic phase: *n*-hexane:ethyl acetate = 8:2 (v/v) containing 1 mmol L^{-1} NAP racemate, aqueous phase: 0.1 mol L^{-1} phosphate buffer solution with pH = 2.67, and temperature was 5 °C.

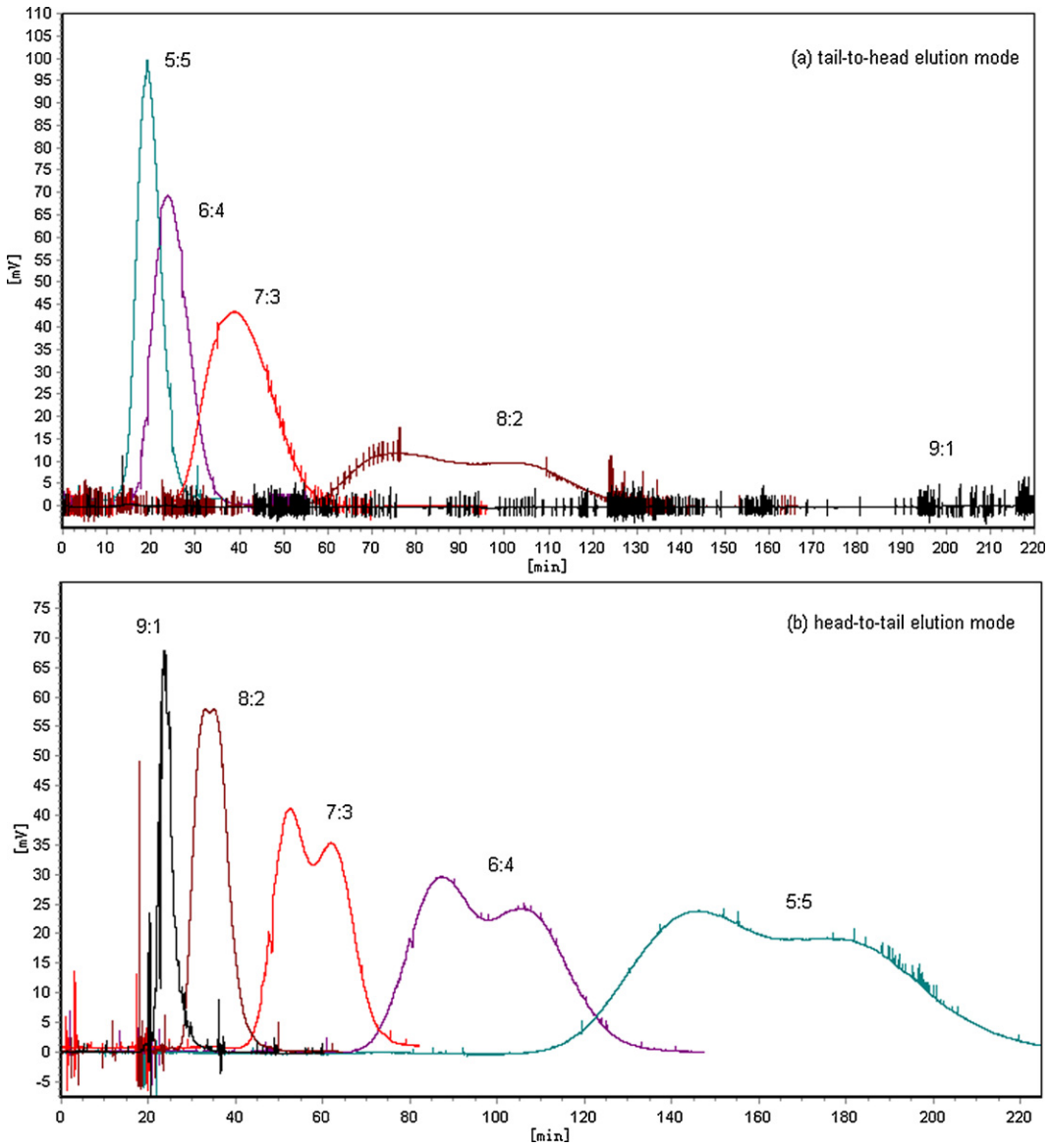

Fig. 3. Chromatogram of analytical chiral HSCCC for the separation of (*R*, *S*)-NAP with different solvent systems. Experimental conditions: solvent system: *n*-hexane:ethyl acetate:0.1 mol L^{-1} phosphate salt buffer solution with pH = 2.67 containing 0.1 mol L^{-1} HP-β-CD (9:1:10, 8:2:10, 7:3:10, 6:4:10 and 5:5:10, v/v/v); stationary phase: (a) lower aqueous phase, (b) upper organic phase; mobile phase: (a) upper organic phase, (b) lower aqueous phase; sample solution: 1.5 mg of NAP racemate dissolved in 1 mL of the organic phase; The molar ratio of HP-β-CD/racemate (a) 230:1 and (b) 138:1 in the chromatographic system; flow rate: 0.5 mL min^{-1}; revolution: 1820 rpm; stationary phase retention: (a) 75% for all the separations; (b) 55% for all the separations.

β-CD:racemate was 230:1. The stationary aqueous phase was 0.1 mol L^{-1} phosphate salt buffer solution (pH = 2.67) added with 0.1 mol L^{-1} HP-β-CD, the following separations were performed on an analytical scale with a two-phase solvent system composed of *n*-hexane–ethyl acetate–aqueous phase (9:1:10, 8:2:10, 7:3:10, 6:4:10 and 5:5:10, v/v/v), Fig. 3(a) shows HSCCC chromatogram for the separations of NAP racemic mixture. As the chromatogram shown, the mobile organic phase (9:1) could not elute the analyte within 220 min and no peak resolution was observed for 5:5, 6:4 and 7:3. The mobile organic phase (8:2) provided visible resolution for NAP enantiomers, but this system also led to too long retention time for enantiomers. Too much long retention time would be resulted if the preparative HSCCC apparatus was employed. Therefore, head-to-tail elution mode was investigated with analytical TBE-20A instruments with the molar ratio of HP-β-CD:racemate was 138:1 (Fig. 3(b)). Retention of organic stationary phase was 55%. Analytical experiment results proved that two solvent systems would be suitable for enantioseparation of NAP racemate: *n*-hexane–ethyl acetate–aqueous phase (8:2:10 and 7:3:10, v/v/v). But the solvent system 7:3:10 led to too much long retention time for NAP analyte if conducted with preparative TBE-300A instrument, as

Table 2
Influence of the temperature on the enantioseparation of NAP enantiomers. Organic phase:*n*-hexane–ethyl acetate (8:2, v/v) containing 1 mmol L^{-1} racemic NAP, aqueous phase: [HP-β-CD] = 0.10 mol L^{-1}, pH = 2.67 and the molar ratio of CS/racemate was 100:1.

Temp. (°C)	D_S	D_R	α
5	0.39	0.56	1.43
10	0.42	0.56	1.36
15	0.46	0.61	1.32
20	0.50	0.63	1.27
25	0.50	0.63	1.24
30	0.55	0.67	1.20
40	0.60	0.70	1.16
50	0.59	0.67	1.12

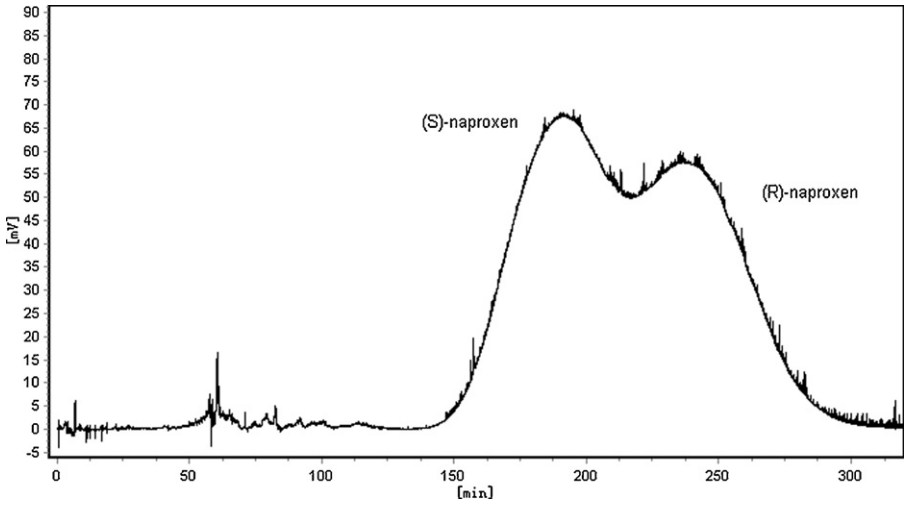

Fig. 4. Chromatogram of preparative chiral HSCCC for separation (*R, S*)-NAP. Experimental conditions: solvent system: *n*-hexane:ethyl acetate:0.1 mol L^{-1} phosphate salt buffer solution with pH = 2.67 (7:3:10, v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; sample solution: 30 mg of NAP racemate dissolved in 20 mL of the organic phase. Flow rate: 2.0 mL min^{-1}; revolution: 850 rpm; stationary phase retention: 60%. The molar ratio of HP-β-CD/racemate 82:1 in the chromatographic system.

shown in Fig. 4, which was not suitable for recycling HSCCC separation. Finally, the solvent system 8:2:10 proved to be suitable for enantioseparation of NAP racemate with suitable retention time.

Fig. 5 shows the typical separations of NAP enantiomer using the preparative HSCCC technique with recycling elution mode and aqueous phase was used as mobile phase. With the solvent systems *n*-hexane–ethyl acetate–aqueous phase (8:2:10), the separations were performed with 29 mg of NAP racemic mixtures. Retention of organic stationary phase was 59% and the molar ratio of HP-β-CD:racemate was 83:1.The sample solution was injected after hydrodynamic equilibrium was reached. After three cycles eluates containing (*S*)-NAP and (*R*)-NAP were collected manually in the individual conical flask according to chromatogram. The separation included four cycles. The first cycle exhibited a fork-like peak and the third cycle almost gave complete separation for NAP enantiomers. At the end of third cycle the switching valve was returned to normal elution and the fourth cycle gave complete separation for enantiomers. The end of (*R*)-NAP peak of the fourth cycle would overlap the front of the next peak (*S*)-NAP of the fifth cycle because the column volume was limited. Recycling HSCCC enantioseparation showed much advantage in achieving effective separations through preventing stationary phase loss. The peak extension between separation cycles might be an impact factor for a successful separation [11]. More separation cycles could be carried out with a shorter peak extension. Therefore in order to shorten the peak extension, it might be useful to use a mobile phase in which the target compounds are easily dissolved. As for recycling chiral HSCCC separation, distribution ratio for enantiomers should be less than 1 so that the target peaks could be limited in a narrow range.

Additional technical problems for successful recycling chiral HSCCC separation might be discussed here. First, the success of recycling is greatly dependent on the volume of tubing connecting the outlet of the detector to the inlet of the pump. The permitted number of cycles in the recycling HSCCC separation would be directly associated with the volume of tubing. In the second place, to selection of time in which the recycling elution was formed need to be considered. Generally, one could choose any time between after sample injection and before the elution of first cycle to begin recycling elution mode. However, during the recycling chiral HSCCC separation of NAP enantiomers, better HSCCC chromatogram was obtained when the recycling elution mode was formed at the time immediately before the first cycle emerged (retention time was 80–90 min). If the recycling elution mode began at the time immediately after sample injection, the HSCCC chromatogram appeared rather unstable. The above phenomenon might be explained by two possible reasons. The first one was that new hydrodynamic equilibrium needs to be reached in the separation column before the elution of the NAP analyte since sample solution was injected. The second one was there might be some hydrophilic impurities existed in the separation column, which will emulsify the two-phase solvent system if they were not eluted out after sample injection. Finally, recycling elution mode always produces broaden peaks. Peaks become much broader with increasing number of cycles. The peak range extended from 50 min in the first cycle to 130 min in the fourth cycle, as shown in Fig. 5. But peaks in the fourth cycle were sharper than the peaks in the second and third cycles. This abnormal phenomenon might be explained as follows: air bubbles were found to easily appear in the aqueous phase solution containing HP-β-CD even if the aqueous solution was degassed by ultrasound for more than 30 min before use. Air bubbles were difficult to eliminate during HSCCC separation, which might decrease pump's working efficiency during recycling elution since bubbles would repeatedly passed through the pump. However, air bubbles eluted from the separation column would not be pumped when recycling elution mode was over after the third cycle and flow rate increased to normal level, which led to sharper peak than that of second and third cycles but it was still broader than that of the first cycle.

As for chiral HSCCC resolution of racemates, it is very important to make sure that the amount of racemate to be injected was set to avoid saturation of the CS in the separation column. A maximum molar ratio CS/analyte (1:1) was believed to the limit capacity in chiral CCC separation, in which CS forms 1:1 complexes with enantiomers. And solubility of the analyte in the two phase solvent system also need consideration. 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 [32]. So the maximum amount of sample that can be dissolved in the organic phase (20 mL for the preparative apparatus) was investigated. The results showed that the maximum amount of sample that could be dissolved in the organic stationary phase was 1.5 mg mL^{-1} (6.5 mmol L^{-1}) under 5 °C. As for the preparative separation with 60% retention of stationary phase, the molar ratio HP-β-CD/analyte would be in the order of 83:1, which was quite adequate to avoid saturation of the chromatographic system.

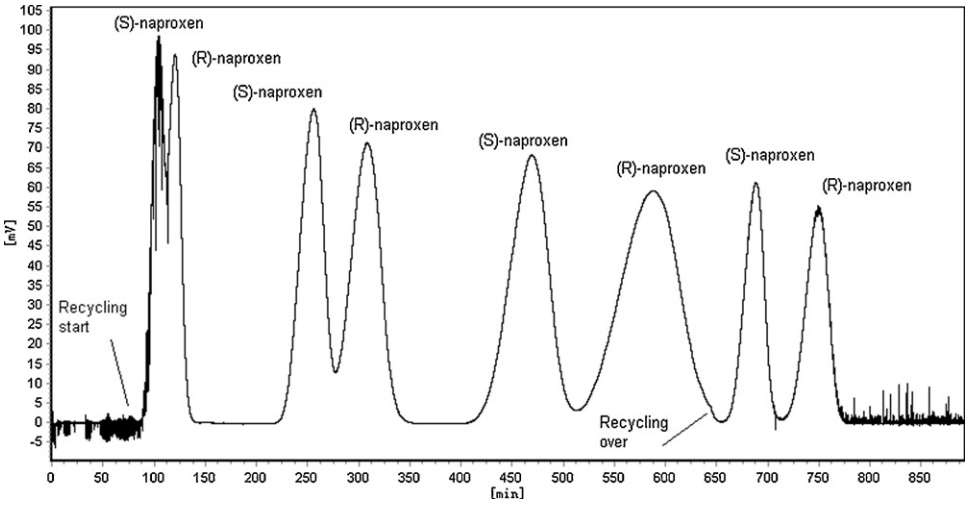

Fig. 5. Separations of (*R*, *S*)-NAP by preparative chiral HSCCC with recycling elution mode. Experimental conditions: solvent system: *n*-hexane–ethyl acetate–0.1 mol L^{-1} phosphate salt buffer solution with pH = 2.67 (8:2:10, v/v/v) containing 0.10 mol L^{-1} HP-β-CD in the aqueous phase; sample solution: 29 mg of NAP racemate dissolved in 20 mL of the organic phase; Flow rate: 2.0 mL min^{-1} in head-to-tail elution mode; revolution: 850 rpm; stationary phase retention: 59%. The molar ratio of HP-β-CD/racemate 83:1 in the chromatographic system; Recycling elution mode was formed at retention time 85 min and was stopped at retention time 640 min.

The racemate of NAP and CCC fractions were analyzed by reverse HPLC with chiral mobile phase additives. The retention time of (*S*)-NAP (t = 36.233 min) is less than that of (*R*)-NAP (t = 40.982 min). HPLC results demonstrated that the purity of both of the (*R*, *S*)-NAP enantiomer were over 99.5% and enantiomeric excess (ee) of (*S*)-NAP and (*R*)-NAP reached 99.4%.

Purification of (*R*, *S*)-NAP enantiomer from HSCCC fractions was carried out by the silica gel column chromatography to remove the small amount of HP-β-CD. Recovery of both of the (*R*, *S*)-NAP enantiomer were in the range of 82–89% and 13 mg of (*S*)-NAP and 12 mg of (*R*)-NAP 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 from the preparative chiral CCC separation was $[\alpha]_D^{30^\circ C}$(methanol) = +44.2, and the optical rotation for the fraction with the more retention time was $[\alpha]_D^{30^\circ C}$(methanol) = −45.0.

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