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Chiral High-Speed Counter-Current Chromatography: Future Strategies for Chiral Selector Development

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Abstract

In conventional high-performance liquid chromatography, chiral separations are performed by chiral column with a chiral selector (CS) chemically bonded to the solid support. In contrast, high-speed counter-current chromatography (HSCCC) performs chiral separations by dissolving CS in the liquid stationary phase. During the past two decades, several CSs were developed to successfully carry out chiral HSCCC which include N-dodecanoyl-L-proline-3,5-dimethylanilide, β -cyclodextrin derivatives, vancomycin, cinchona alkaloid derivatives, cellulose and amylose derivatives, tartaric acid derivatives, etc. Compared to HPLC which uses over hundred different kinds of CSs, the number of CSs effectively used in HSCCC is limited to several compounds. This may be due to the violent molecular movement of CS dissolved in the liquid stationary phase which reduces chiral selectivity based on steric affinity. Future development strategy of CS for HSCC proposed here is to suppress the molecular movement of the CS in the liquid stationary phase by the following three ways: 1) using viscous stationary phase such as aqueous-aqueous polymer phase system; 2) attaching a long hydrophobic chain to the asymmetric carbon, or 3) chemically bonding CS onto hydrophobic small particles such as carbon nanotubes, gold colloidal particles, and submicron silica particles.

Keywords

chiral separation; high-speed counter-current chromatography; chiral selector; steric interaction

1. Introduction

For many decades separations of enantiomers have been widely performed by conventional high-performance liquid chromatography (HPLC), SFC and electrophoresis. In the HPLC method a suitable chiral selector (CS) is chemically bonded to a solid support to separate enantiomers according to their affinity to CS. Currently over a number of different chiral columns are available in the market. The method, however, requires high cost of immobilizing the CS onto the solid support, and the amount of yield is limited. For pharmaceutical applications, a preparative scale of chiral compounds is required. High-speed counter-current chromatography (HSCCC) is a unique member of liquid partition technique which does not utilize the solid support, hence the sample loss due to irreversible

adsorption and degradation over the solid/liquid interface is prevented [1, 2]. In addition, it retains a large volume of stationary phase which permits separation of a gram quantity of samples in a small separation column. The method uses a planetary centrifuge to rotate a coiled separation column under a rotating centrifugal force field. This produces an Archimedean screw effect to mix the two phases while retaining a large volume of the stationary phase in the column under a relatively high flow rate of the mobile phase. The method can perform an efficient chiral separation by simply dissolving a suitable CS in the liquid stationary phase. Since 1995, several papers on chiral CCC have been published using a variety of CSs including N-dodecanoyl-L-proline-3,5-dimethylanilide [3-6], β -cyclodextrin derivatives [7,8], vancomycin [9], cellulose and amylose derivatives [10], cinchona alkaloid derivatives [11], (+)-(18-crown-6) tetracarboxylic acid [12], (S)-naproxen derivatives [13], tartaric acid derivatives [14] as listed in Table 1. However, compared with the number of chiral selectors in HPLC, the number of CSs so far developed for HSCCC is no more than one tenth of those currently available for HPLC separations. In this review paper, chiral selectors so far used for HSCCC are reviewed and the strategies of future development of CSs for HSCCC are discussed.

2. N-dodecanoyl-L-proline-3,5-dimethylanilide

This CS introduced by Pirkle in 1981 [15] has been applied by Foucault et al. for separation of enantiomers of DNB-valine and leucine in centrifugal partition chromatography in 1995 [16]. In 1996 it was first applied to HSCCC by Ma et al. for the separation of a variety of DNB-amino acids [3-6]. Using this CS a series of basic studies have been performed for both analytical and preparative scale separations to optimize experimental conditions for chiral HSCCC.

2.1. Analytical scale separations

2.1.1. Effect of chain length attached to the asymmetric carbon—The separations were performed using both our prototype fabricated in the National Institutes of Health Machine Shop and a commercial model purchased from P.C. Inc. (Potomac, MD, USA). The separations were performed as follows: The column is first entirely filled with the stationary phase which contains a desired amount of CS while leaving about 10% of CS-free stationary phase at the end of the column (this prevents carryover of the CS in the fractions). After sample injection, the mobile phase is pumped into the column at a desired flow rate while the column is rotated at 800 rpm. The separations are carried out by successive injection of the sample solution without renewing the stationary phase containing CS in the column.

Figure 1 shows the separation of four pairs of DNB-amino acids by the standard CCC technique using a two-phase solvent system composed of hexane-ethyl acetate-methanol-10 mM HCl at a volume ratio of 8:2:5:5 and N-dodecanoyl-L-proline-3,5-dimethylanilide as a CS [3]. Because of its high hydrophobicity, CS was almost entirely retained in the upper organic stationary phase ($K > 100$) and all components were well resolved in 1-3 hours. As clearly shown from the chromatograms, peak resolution of DNB-phenylalanine is much greater than that of DNB-phenylglycine suggesting that the longer hydrocarbon chain attached to the asymmetric carbon increases the retention time. Similarly the resolution between DNB-leucine racemate is far greater than that between DNB-valine racemates (Table 2). These results indicate that the chain length of the hydrocarbon attached to asymmetric carbon may produce an important effect on chiral selection. Two other CSs were also tested by HSCCC: N-2',6'-dimethylpiperidine-6-methyl- α -naphthalene ethanamide and N-dodecanoyl-6-methoxy- α -methyl-2-naphthalene ethanamide. The DNB-leucine enantiomers were resolved by both CSs with the same solvent system (Figs. 2A & B). An increased length of the side chain at asymmetric center of CS not only alters the

separation factor but also increases the hydrophobicity of CS to promote partition toward the organic stationary phase.

All CSs successfully used in the above studies are similar to the chiral stationary phase that has been introduced by Perkle et al. for the HPLC separation of racemic DNB-amino-acid [9]. This may indicate that the CSs used as the HPLC solid stationary phase can also be efficiently utilized for HSCCC in their free form, i.e., without immobilization to the solid support.

2.1.2. Effect of CS concentration in the stationary phase—A series of experiments was performed to investigate the effects of the CS concentration in the organic stationary phase on chiral resolution. Fig. 3 shows a set of chromatograms of 4 DNB-amino acid racemates obtained by introducing various amounts of CS in the stationary phase [4]. In the CS-free separation (top, row), all racemates formed a single peak as expected and eluted shortly after the mobile phase front with similar retention times. As the amount of CS was increased, each racemate was resolved where (-)-enantiomers with less affinity to the CS eluted earlier, whereas (+)-enantiomers with a stronger affinity to CS retained longer in the column.

Fig. 4 illustrates the effects of the amounts of CS in the stationary phase on the separation factor (4A) and peak resolution (10B) [4]. The separation factors of all racemates became greater as the amount of CS in the stationary phase was increased from 0 to 4 g where they reached near saturation (Fig. 4A). The effects of the amount of CS on the resolution are similarly shown in Fig. 4B. In contrast with the separation factor (Fig. 4A), peak resolution for all of the racemates increased nearly in proportion to the net amount of CS up to 4 g indicating that the resolution can be improved by further increasing the CS concentration in the stationary phase. The above results clearly indicate an important strategy for the present method, i.e., the best peak resolution is attained by saturating the CS in the stationary phase in a given column, and the resolution is further improved by using a longer and/or wider bore coiled separation column that can hold a greater amount of CS in the stationary phase.

The effect on the CS distribution within the stationary phase was also studied. A given amount of CS was dissolved in various volumes of the stationary phase ranging from 50 to 200 ml so that the CS concentration was increased while the amount of CS remained constant. These CS containing stationary phase were each placed at the beginning portion of the column and the separation was performed with the same solvent system used in the previous experiment. Fig. 5 illustrates a set of chromatograms of four DNB-amino acid racemates according to the similar format used in Fig. 3 [4]. The results clearly showed that the retention times of all four racemates were almost identical regardless of the volume of the stationary phase to which the CS was dissolved, indicating that the net amount of CS determines their α values. The peak resolution, on the other hand, was found to vary according to the CS distribution in the column. The 50-ml group shows substantially lower resolution than the 200-ml group. This may be explained as follows: It can be considered that the two columns are connected in series. The first column is the chiral-active column and the second is a classical achiral column. The 50-ml group is subjected to chiral separation in a four-fold shorter than that of the 200-ml column. The retention times and separation factors are identical because the solutes have been exposed to the same mass of CS. The resolution factors differ because the effective column lengths are different.

2.1.3. Effect of hydrophobicity of the solvent system—In this series of experiments, the hydrophobicity of the two-phase solvent system composed of hexane/ethyl acetate/methanol/10 mM HCl was modified by changing the volume ratio between hexane and ethyl acetate, while the volume ratio of methanol and 10 mM HCl was kept constant. In

all experiments, a given amount (1 g) of CS was dissolved in 200 ml of organic stationary phase. The results of the experiments are illustrated in Fig. 6 [4]. As expected, the retention times of both DNB-valine and DNB-leucine racemates increased steadily with the reduced hydrophobicity of the solvent system when the hexane/ethyl acetate volume ratio was decreased as indicated in the left margin.

The separation factor and peak resolution for these separations are graphically illustrated in Fig. 7 where these two parameters are plotted against the hexane/ethyl acetate volume ratio [4]. Quite different curves are observed between these two parameters. The α -values of both racemates decrease sharply as the hydrophobicity of the solvent decreases. The R values of these two racemates, on the other hand, show convex curves each with a maximum value at a different locations: DNB-leucine maximum value is at 9:1 volume ratio whereas of more polar DNB-valine is around 7:3 while in both cases the mean partition coefficient of the racemates is about 0.7. These results indicate that the best peak resolution is attained by optimizing the hydrophobicity of the solvent system so that the mean partition coefficient value for each racemate falls in a suitable ranges, e.g., between 0.6 and 0.8 in the above case. Although the use of the hydrophobic solvent system may yield a higher α value for the racemate, the peak resolution could be reduced due to an excessively short retention time.

2.2. Preparative separation of enantiomers

2.2.1. Standard CCC—The preparative capability of the present system was investigated on the separation of DNB-leucine enantiomers by varying the CS concentrations from 10 to 60 mM in the solvent system composed of hexane/ethyl acetate/methanol/10 mM HCl (6:4:5:5, v/v). The results shown in Fig. 8 indicate that the sample loading capacity is mainly determined by the concentration (or net amount) of CS in the stationary phase [3], i.e., the higher the CS concentration (or amount) in the stationary phase, the greater the peak resolution and the sample loading capacity. The maximum sample size of 1 g was completely resolved in 9 hours with a 330 ml capacity column, the same column used for the analytical separation. The above results indicate that the standard HSCCC column can be used for both analytical and preparative separations simply by adjusting the amount of CS in the stationary phase. If the CS molecule is stable enough, the method has an additional advantage in that the column can be used repeatedly to separate a variety of enantiomers by dissolving appropriate CSs in the stationary phase. The larger scale separation can be achieved by using a longer and/or greater inner diameter coiled column and also by applying the pH-zone-refining CCC technique described below.

2.2.2. pH-zone-refining CCC—pH-zone-refining CCC is a powerful preparative technique that is comparable to displacement chromatography and isotachopheresis. It yields a succession of highly concentrated rectangular solute peaks with minimum overlap where impurities are concentrated at the peak boundaries [17, 18]. This technique was applied to the resolution of DNB-($\pm$)-leucine racemates using a binary two-phase solvent system composed of methyl *tert*.-butyl ether/water where trifluoroacetic acid (retainer) and N-dodecanoyl-L-proline-3,5-dimethylanilide (CS) were added to the stationary phase and ammonia (eluter) to the aqueous mobile phase.

Fig. 9 shows a typical chromatogram obtained by pH-zone-refining CCC, where a 2 g amount of ($\pm$)-leucine was eluted in a single rectangular UV (solid line) in about 3 hours [3]. The pH of the obtained fractions (dotted line) revealed that the peak was evenly divided into two pH zones with a sharp transition. When peak fractions were analyzed by the analytical-scale standard CCC technique described earlier, the first zone (pH 6.5) was almost entirely composed of (-)-DNB-leucine and the second zone (pH 6.8) of (+)-DNB-leucine, whereas

the narrow zone boundary contained both isomers and an impurity (upper diagram). The mixing zone is estimated to be no more than 5% of each peak.

Compared with the standard CCC technique described earlier, the pH-zone-refining CCC technique allows separation of larger amounts in shorter separation times. In addition, the method uses relatively polar solvent systems that can hold the CS for a much longer time within the column, reducing contamination in the purified fractions. In both techniques leakage of CS in the eluate can be completely eliminated by filling the outlet of the column with a proper amount of CS-free stationary phase so as to absorb CS molecules leaking into the flowing mobile phase.

3. β -Cyclodextrin derivatives

β -Cyclodextrin derivatives can form various aqueous solutions and uses as an effective CS in HPLC chiral separation [16]. Recently, they are used for chiral separation by HSCCC in two research groups [7, 8]. Fig. 10 shows a set of chromatograms of racemic lomefloxacin hydrochloride obtained by different concentrations of CS in the two-phase solvent system composed of ethyl acetate-methanol-water at a volume ratio of 10:1:10. These chromatograms are arranged from a to c according to the amount of sample ranging from 5 mg/4 ml to 20 mg/4 ml, and from b to i according to the amount of CS in the solvent systems ranging from 0 to 60 mg/l. In the CS-free separation (0 mg/l) the racemic lomefloxacin hydrochloride only gave a single peak as expected. As the concentration of CS was increased, the racemate was resolved into their isomers where (-)-enantiomer was eluted earlier than (+)-enantiomer. Increasing the concentration of CS in the solvent systems tends to decrease the retention time of the first peak. This may be due to the fact that CS is more strongly combined with (-)-enantiomers than (+)-enantiomers [7]. Fig. 11 shows separation of α -cyclohexyl mandelic acid (α -CHMA) enantiomers using a two-phase solvent system composed of hexane-methyl *tert*.-butyl ether-0.1 M phosphate buffer solution (pH 2.68) (9:1:10, v/v) containing two chiral selectors, 0.3 M (-)-2-ethylhexyl tartarate in the organic stationary phase and 0.1M hydroxypropyl β -cyclodextrin in the lower aqueous mobile phase. Using an HSCC instrument with a preparative column with a 260 ml capacity (Shanghai Tauto Biotech, Shanghai, China) the separation was performed at a flow rate of 2 ml/min. A 440 mg amount of racemates was completely resolved in 7 hours.

4. Other chiral selectors for HSCCC

Besides the above described, several other CSs, including vancomycin [9], cinchona alkaloid derivatives [11], cellulose and amylose derivatives [10], (+)-(18-crown-6) tetracarboxylic acid [12] and (S)-naproxen derivatives [13], have been developed and successfully used for chiral separations. Using vancomycin, Duret et al. [9] resolved dancyl-nor-leucine using an analytical HSCCC column (13 ml capacity) with a two-phase solvent system composed of toluene-aqueous solution containing 140 mg/mg of vancomycin (pH 4.7) using a dual elution mode where the D-enantiomer was strongly retained in the aqueous phase. When L-enantiomer was eluted, the mobile phase was switched to the other phase to elute D-enantiomer which was retained in the column.

5. Future development of CS for HSCCC

Compared with the conventional HPLC technique which uses over a hundred of different chiral selectors, the number of effective CS available for HSCCC is limited to no more than several compounds. The reason may be speculated as follows: Affinity between two molecules may be generated by several mechanisms including electric charge interaction, hydrogen bond, hydrophobic interaction, π -electron interaction, steric interaction, etc. Among those, steric interaction like chiral affinity is considered to be highly sensitive to the

motion of molecules in the solvent (source of Brownian motion). In the conventional chiral column, the CS is immobilized onto the solid support to interact with the racemates in the mobile phase whereas in HSCCC the CS is freely moving around in the liquid stationary phase making harder to meet two molecules at an appropriate steric position. This hypothesis is supported by the result of our studies that the hydrocarbon chain directly connected to the asymmetric carbon is affected on the chiral selectivity: the longer is the hydrocarbon chain, the better chiral separation is obtained [4]. It is also shown that lower temperature that suppresses the molecular motion improves the chiral selectivity while the partition efficiency is decreased [19]. Majority of CSs for HSCCC reported such as sulfated β -cyclodextrin [7,8], vancomycin [9], bovine serum albumin [20, 21], cellulose and amylose derivatives [11] and ($\pm$)-(18-crown-6)-tetracarboxylic acid [12] have a relatively high molecular weight which reduces molecular movement in the stationary phase. On the basis of the above finding, more CS for HSCCC may be developed by suppressing the molecular motion of the CS in the liquid stationary phase. This may be achieved by the following methods: First, the use of viscous two-phase solvent system such as polyethylene glycol (PEG)/phosphate and PEG/dextran aqueous-aqueous systems may improve the chiral separation by suppressing the molecular motion of CS in the upper PEG-rich phase or lower dextran-rich phase [22]. Next, the long hydrocarbon chain is attached to the asymmetric carbon, which not only enhances the chiral affinity but also improves the retention of the CS in the stationary phase. The molecular motion of CS is further suppressed by attaching the CS to small particles such as carbon nano-tube or gold colloidal nano-particles, or even submicron silica particles which cannot be used as a solid support in the conventional HPLC technique.

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Figure 1.

Analytical separation of four DNB-amino acid racemates by HSCCC.

Experimental conditions: Apparatus: multilayer coil HSCCC centrifuge with a semi-preparative column of 1.6 mm I.D. and a 330 ml capacity; solvent system: hexane/ethyl acetate/methanol/10 mM HCl (8:2:5:5, v/v), N-dodecanoyl-L-proline-3,5-dimethylanilide (CS) was added to the organic stationary phase (200 ml); sample: 5-10 mM of each DNB-amino acid dissolved in 5 ml of solvent consisting of equal volumes of each phase; flow rate: 3.3 ml/min in the head - tail elution mode; revolution: 800 rpm; stationary phase retention: 65%.

Figure 2.

Analytical separations of DNB-leucine enantiomers using naproxen derivatives as the chiral selector.

Experimental conditions: triplet coil planet centrifuge for HSCCC equipped with a set of multilayer coil with 0.85 mm I.D. and 170 ml capacity; solvent system: hexane/ethyl acetate/methanol/10 mM HCl (7:3:5:5, v/v), N-dodecanoyl-6-methoxy- α -methyl-2-naphthalene ethanamide (10 mM) (A) and N'-(2',6'-dimethylpiperidine)-6-methoxy- α -methyl-2-naphthalene ethanamide (20 mM) (B) were added to the organic stationary phase (80 ml) as CS; sample: 2 mg ($\pm$)-DNB-leucine dissolved in 1 ml of lower phase; flow rate: 1 ml/min; revolution: 1000 rpm; stationary phase retention: 42% (A) and 57% (B). [23]

Figure 3.

Effects of the amount or concentration of CS on the separation of DNB-amino acid racemates.

In all resolved chromatograms, the first peak represents (-)-enantiomer and the second peak (+)-enantiomer. Experimental conditions: commercial HSCCC centrifuge (Ito multilayer coil separator/extractor) with 10 cm revolution radius; separation column: a multilayer coil consisting of 1.6 mm I.D. PTFE tubing with a total capacity of 330 ml; sample: DNB-amino acid mixture consisting of DNB-phenylglycine, DNB-phenylalanine, DNB-valine, DNB-leucine each 5-10 mg dissolved in 2 ml solvent (1 ml each phase); solvent system: hexane/ethyl acetate/methanol/10 mM HCl (8:2:5:5, v/v); stationary phase: upper phase with CS ranging from 0 -4 g in 200 ml as indicated; mobile phase: lower aqueous phase; flow rate: 3 ml/min; revolution: 800 rom.

Figure 4. Effects of net amount of CS on the selectivity factor, α (A) and the resolution factor, R , (B) of DNB-amino acid racemates.

Figure 5.

Effects of CS distribution in the stationary phase on the separation of DNB-amino acid racemates.

Experimental conditions: Apparatus: commercial HSCCC centrifuge with 10 cm revolution radius; separation column: multilayer coil of 1.6 mm I.D. Tefzel tubing with 320 ml capacity; sample: ($\pm$)-DNB-valine and ($\pm$)-DNB-leucine each 5-10 mg; solvent system: hexane/ethyl acetate/methanol/10 mM HCl (8:2:5:5, v/v); stationary phase: upper organic phase containing 1 g of CS in 50, 100, and 200 ml; mobile phase: lower aqueous phase: flow rate: 3 ml/min; revolution: 800 rpm.

Figure 6.

Effects of hydrophobicity of the solvent system on separation of DNB-amino acid racemates.

Experimental conditions: Sample: ($\pm$)-DNB-valine and ($\pm$)-DNB-leucine each 5-10 mg; solvent system: hexane/ethyl acetate/methanol/ethyl acetate/methanol/10 mM HCl at volume ratios of 10:0:5:5, 10 mM HCl; 10 mM HCl at volume ratios of 10:0:5:5, 10 mM HCl at volume ratios of 10:0:5:5, 10 mM HCl at volume ratios of 10:0:5:5, 10 mM HCl at volume ratios of 10:0:5:5, 9:1:5:5, 8:2:5:5, 7:3:5:5, 6:4:5:5, 5:5:5:5 from the top to the bottom rows. In each solvent system 1 g of CS was introduced in 200 ml organic stationary phase. Other experimental conditions are identical to those described in Fig. 1.

Figure 7. Effects of hydrophobicity of the solvent system on the α and R values of the racemates of DNB-valine and DNB-leucine. Experimental conditions are described in Fig. 4.

Figure 8.

Preparative separation of (±)-DNB-leucine by the standard HSCCC technique.

Experimental conditions: Apparatus: same as described in Fig. 1; solvent system: hexane/ethyl acetate/methanol/10 mM HCl (6:4:5:5, v/v) where the organic stationary phase containing CS at 10 – 60 mM; sample: (±)-DNB-leucine, 25 – 1000 mg dissolved in 10-45 ml of solvent consisting of equal amounts of each phase; flow rate: 3.3 ml/min; revolution: 800 rpm; retention of stationary phase: 64%.

Figure 9. Chiral separation of (±)-DNB-leucine racemates by pH-zone-refining CCC

Experimental conditions: Apparatus: same as used in Fig. 1 experiment; solvent system: methyl *tert.*-butyl ether/acetonitrile/water (4:1:5, v/v); stationary phase: upper organic phase containing 40 mM CS and 40 mM (A) or 80 mM (B) trifluoroacetic acid (TFA); lower aqueous phase to which ammonium hydroxide was added at 20 mM; sample: 2 g of (±)-DNB-leucine was dissolved in 50 ml solvent consisting of equal volumes of each phase; flow rate: 3.3 ml/min; revolution: 800 rpm; analysis: chirality by analytical HSCCC and pH by a portable pH meter. Other experimental conditions are described in Fig. 1 caption. Stationary phase retention: 65%.

Fig 10.

Effects of the concentration of CS and the amount of sample on the separation of racemic lomefloxacin hydrochloride.

Apparatus: HSCCC with multilayer coil consisting of 1.6mm I.D. PTFE tubing with a total capacity of 150 ml; sample: racemic lomefloxacin hydrochloride 5–20 mg dissolved in 4ml of solvent (2 ml of upper phase and 2ml of lower phase); solvent system: ethyl acetate–methanol–water (10:1:10, v/v), different CS concentrations (0, 10, 20, 40, 50 and 60 mmol/l) in this solvent systems; stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 1ml/min; revolution speed: 800 rpm. In all resolved chromatograms, the first peak represents (–)-enantiomers and the second peak, (+)-enantiomers.

Fig. 11.

Separations of ($\pm$)- α -CHMA by preparative chiral CCC technique.

Experimental conditions: column volume 260 ml; solvent system: n-hexane–methyl *tert.*-butyl ether–0.1 mol/l phosphate salt buffer solution with pH= 2.68 (9:1:10, v/v/v) containing 0.3 mol/l (–)-2-ethylhexyl tartarate in the organic phase and 0.1 mol l^{–1} HP- β -CD in the aqueous phase; sample: 440mg 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.

Table 1
Chiral selectors, enantiomers and solvent systems used in chiral CCC

Chiral selector	Enantiomer	Two-phase-solvent system (volume ratio)	Reference
N-dodecanoyl-L-proline-3,5-dimethylanilide	DNB-amino acid racemates	Hex-EtOAc-MeOH-10mM HCl (8:2:5:5)	[3-6]
β-cyclodextrin derivatives	α-cyclohexylmandelic acid	Hex-MtBE-H ₂ O (9:1:10)	[7]
	Lomefloxacin hydrochloride	EtOAc-MeOH-H ₂ O (10:1:10)	[8]
	Phenylsuccinic acid	Hex-MtBE-0.1M phosphate buffer (1:3:4)	[20]
Cinchona alkaloid derivatives	N-(3,5-DNB)-(+/-)-leucine	Hep- <i>tert.</i> -AmOH-MeOH-AA buffer (10:5:1:5)	[11]
Vancomycin	Dancyl norleucine	toluen-buffer (pH 4.7)(1:1)	[9]
Cellulose and amylose arylcarbamate	Pindolol	MtBE-50mM sodium phosphate buffer (1:1)	[10]
	Warfarin	MtBE-10mM TFA (1:1)	[10]
(+)-(18-crown-6) tetracarboxylic acid	Gemifloxacin	BuOH-EtOAc-20mM Tris buffer (6:5:10)	[12]
(S)-naproxen N,N-diethylamide	(+/-)-2 racemates	Hex-EtOAc-MeOH-H ₂ O (9:1:9:1)	[13]
	(+/-)-3 racemates	MtBE-50 mM sodium phosphate buffer (1:1)	[13]
L-(+)-tartaric acid	D,L-methylbenzylamine	CHCl ₃ -MeOH-H ₂ O (4:3:1)	[14]

DNB: dinitrobenzoyl; Hex: hexane; Hep: heptane; EtOAc: ethyl acetate; MeOH: methanol; MtBE: methy tert.-butyl ether; AA: ammonium acetate; BuOH: 1-butanol; TFA: trifluoroacetic acid; AmOH: amyl alcohol; Tris: bis(2-hydroxyethyl) methane acetate; (+/-)-2 racemates: ((+/-)-2 - N-(3,4-cis-3-decyl-1,2,3,4-tetrahydrophenanthren-4-yl)-3,3-dinitrobenzamide: (+/-)-3: (N-(3,5-dinitrobenzoyl) -(+/-)-leucine

Table 2
Effects of CS distribution on separation of DNB-amino acid racemates

Racemic samples	CS ¹ containing	Stationary phase(mL)	Retention time(mins) ²		α ³	R_s ⁴
			A-	A+		
DNB-phenylglycine	50		35	44	1.60	0.8
	100		36	46	1.55	1.2
	200		36	46	1.59	1.3
DNB-phenylalanine	50		44	64	1.80	1.8
	100		42	61	1.84	2.0
	200		43	64	1.88	2.2
DNB-valine	50		35	47	1.71	1.2
	100		35	46	1.68	1.4
	200		35	44	1.65	1.5
DNB-Leucine	50		53	106	2.56	3.0
	100		54	110	2.60	3.5
	200		50	98	2.55	3.7

¹ CS, chiral selector, N-dodecanoyl-L-proline-3,5-dimethylamide.

² A-,A+, (-) and (+)-3,5-dinitrobenzoyl amino acid.

³ α , Separation factor.

⁴ R_s , peak resolution