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Resolution of gram quantities of racemates by high-speed counter-current chromatography

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Abstract

Gram quantities of (±)-dinitrobenzoyl amino acids were separated by high-speed counter-current chromatography (CCC) using N-dodecanoyl-L-proline-3,5-dimethylanilide as a chiral selector (CS). Standard and pH-zone-refining CCC techniques were compared. By using the standard technique, 10 mg to a maximum of 1 g of samples was resolved in 2–9 h simply by increasing the concentration of the CS in the stationary phase. By using pH-zone-refining CCC, even more sample (2 g) was efficiently separated in less time (3 h). In both techniques, leakage of CS from the column was negligible. The method requires no solid support and the same column can be used repeatedly to separate a variety of enantiomers by dissolving appropriate chiral selectors in the stationary phase.

1. Introduction

Because of increasing demands for chiral purity, there is intensive interest in techniques for the separation of enantiomers. Direct separation of enantiomers by high-performance liquid chromatography (HPLC) is now widely used and more than one hundred chiral stationary phases are commercially available for analytical-scale separations [1]. However, few preparative applications using HPLC [2] have been reported because of the limited capacity of the standard-size columns and the high cost of solid stationary phases for large columns. Counter-current chromatography (CCC) [3–7] is an excellent alternative since the column requires no solid support and can be used to separate a variety of enantiomers by adding a suitable chiral selector to the stationary liquid phase.

In the past, CCC separations of chiral compounds have been described using various techniques such as droplet CCC [8], rotation locular CCC [9] and centrifugal partition chromatography [10,11]. None of these techniques, however, was suitable for preparative-scale separations in terms of sample size, resolution or separation time.

In our laboratory, highly efficient preparative-scale separations of chiral compounds were achieved by high-speed CCC using both standard [12] and pH-zone-refining CCC [13–16] techniques. We have chosen a chiral selector, N-

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dodecanoyl-L-proline-3,5-dimethylanilide, described by Pirkle and Murray [17] for use in HPLC, and recently introduced as a free molecule for chiral resolution in centrifugal partition chromatography by Oliveros et al. [10]. A series of (±)-3,5-dinitrobenzoyl (DNB) amino acids was used to evaluate the CCC procedures.

2. Experimental

2.1. Reagents

Glass-distilled HPLC-grade organic solvents including methyl *tert.*-butyl ether, hexane, ethyl acetate, methanol and trifluoroacetic acid (TFA) were purchased from Burdick & Jackson Labs., Muskegon, MI, USA. Hydrochloric acid (Fisher Scientific, Fair Lawn, NJ, USA) and aqueous ammonia (>28%) (Baker, Phillipsburg, NJ, USA) were analytical grade. (±)-DNB-leucine and (±)-DNB-phenylalanine were purchased from Aldrich, Milwaukee, WI, USA. Other DNB-amino acids and N-dodecanoyl-L-proline-3,5-dimethylanilide (chiral selector or CS) were synthesized according to the method described by Oliveros et al. [10].

2.2. Apparatus

The experiments were performed using a commercial high-speed CCC centrifuge (Ito multilayer coil separator/extractor; P.C. Inc., Potomac, MD, USA; a comparable instrument is available from Pharma-Tech Research Corp., Baltimore, MD, USA. General features of the apparatus are described elsewhere [12]. The apparatus holds a multilayer coil separation column on the rotary frame at a distance of 10 cm from the central axis of the centrifuge. A counterweight is mounted on the opposite side for balancing the column. The desired planetary motion of the column was produced by coupling a plastic gear mounted on the column holder to an identical stationary gear on the centrifuge axis; the column holder undergoes a synchronous planetary motion, i.e. one rotation about its own axis during one revolution around the central axis of the centrifuge.

The column was prepared in our laboratory by winding a single piece of 160 m × 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing around the holder hub making 11 coiled layers between a pair of flanges spaced 5 cm apart. The total column volume measured 330 ml. The ends of the coil were connected to flow tubes (0.85 mm I.D. PTFE) that enter and exit the centrifuge through its hollow central stationary pipe. As described earlier [12], these flow tubes are twist-free when the column is rotated so that the elution can be performed through the rotating column without the use of rotary seals.

2.3. Preparation of two-phase solvent systems and sample solutions

Three different solvent systems were prepared, two for the standard CCC and one for pH-zone-refining CCC. For standard CCC, solvent systems consisting of hexane–ethyl acetate–methanol–10 m*M* hydrochloric acid (8:2:5:5 and 6:4:5:5, v/v) were used. Each solvent mixture was thoroughly equilibrated in a separatory funnel, and the two phases were separated shortly before use. The chiral selector (CS) was added to the organic stationary phase at various concentrations ranging from 10 to 60 m*M*.

For pH-zone-refining CCC, the desired volumes of methyl *tert.*-butyl ether and water were equilibrated in a separatory funnel. After the two phases were separated, both TFA (20 m*M*) and CS (10–60 m*M*) were added to the organic stationary phase and aqueous ammonia (20 m*M*) was added to the aqueous mobile phase.

The sample solutions were prepared by dissolving a desired amount of the sample in the two-phase solvent system used for separation.

2.4. Separation procedure

Both standard and pH-zone-refining CCC were performed using an apparatus schematically illustrated in Fig. 1. In each separation, the column (a) was entirely filled with the organic stationary phase as follows: about 150 ml of

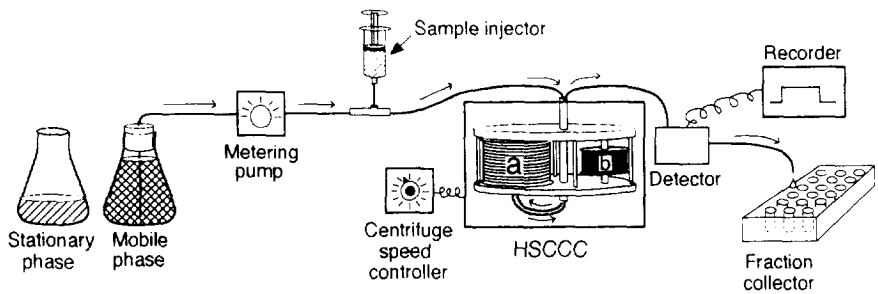

Fig. 1. Elution system for high-speed counter-current chromatography.

CS-free organic phase was first pumped into the inlet of the column (330 ml capacity). Then, 200 ml of the CS-containing organic phase were pumped into the column, discharging ca. 20 ml of excess CS-free stationary phase from the column outlet. The column therefore contained 130 ml of CS-free stationary phase at its outlet. (During the separation, a portion of this CS-free stationary phase was retained in the column to absorb any CS that may be carried over by the mobile phase, thus preventing contamination of the fractions with the CS). After the sample solution was injected through the sample port, the aqueous mobile phase was pumped into the column at a flow-rate of 3.3 ml/min while the column was rotated at 800 rpm regulated by the speed controller. The absorbance of the effluent was continuously monitored at 254 nm (standard CCC technique) or 206 nm (pH-zone-refining CCC) (Uvicord S; LKB, Bromma/Stockholm, Sweden) and 3.3-ml fractions were collected (Ultrorac, LKB).

2.5. Analysis of CCC fractions

Each peak fraction obtained from the standard CCC elution was analyzed by circular dichroism (CD) and/or optical rotation apparatus to determine the chirality. The analytical standard CCC technique thus developed was in turn used for determining optical purity and chirality of fractions obtained from the pH-zone-refining CCC technique. The pH of the fractions from the pH-zone-refining CCC separation was measured (Accumet portable laboratory pH meter, Fisher Scientific, Pittsburgh, PA, USA).

3. Results and discussion

Fig. 2A shows the separations of enantiomers of four different (±)-DNB-amino acids using the standard CCC technique. With a two-phase solvent system composed of hexane–ethyl acetate–methanol–10 m*M* hydrochloric acid (8:2:5:5, v/v), the separations were performed on an analytical scale (5–10 mg for each racemic mixture) to investigate the mechanism of enantioselectivity of the chiral selector. These chromatograms were obtained from left to right by injecting racemic mixtures of DNB-phenylglycine, DNB-phenylalanine, DNB-valine and DNB-leucine, respectively. All of these separations were carried out by injection of successive samples, without renewing the column phases containing the chiral selector. The results indicate that the enantioselectivity of DNB-amino acids is closely related to the side chain (R) of the molecule. Within a class (aliphatic or aromatic) the chromatographic separation factor (α) increases with the increasing size of R as shown in Table 1.

The preparative capability of the present system was investigated with the separation of (±)-DNB-leucine by varying the concentration of the chiral selector from 10 to 60 m*M* in the stationary phase using a similar two-phase solvent system composed of hexane–ethyl acetate–methanol–10 m*M* HCl (6:4:5:5, v/v). The results

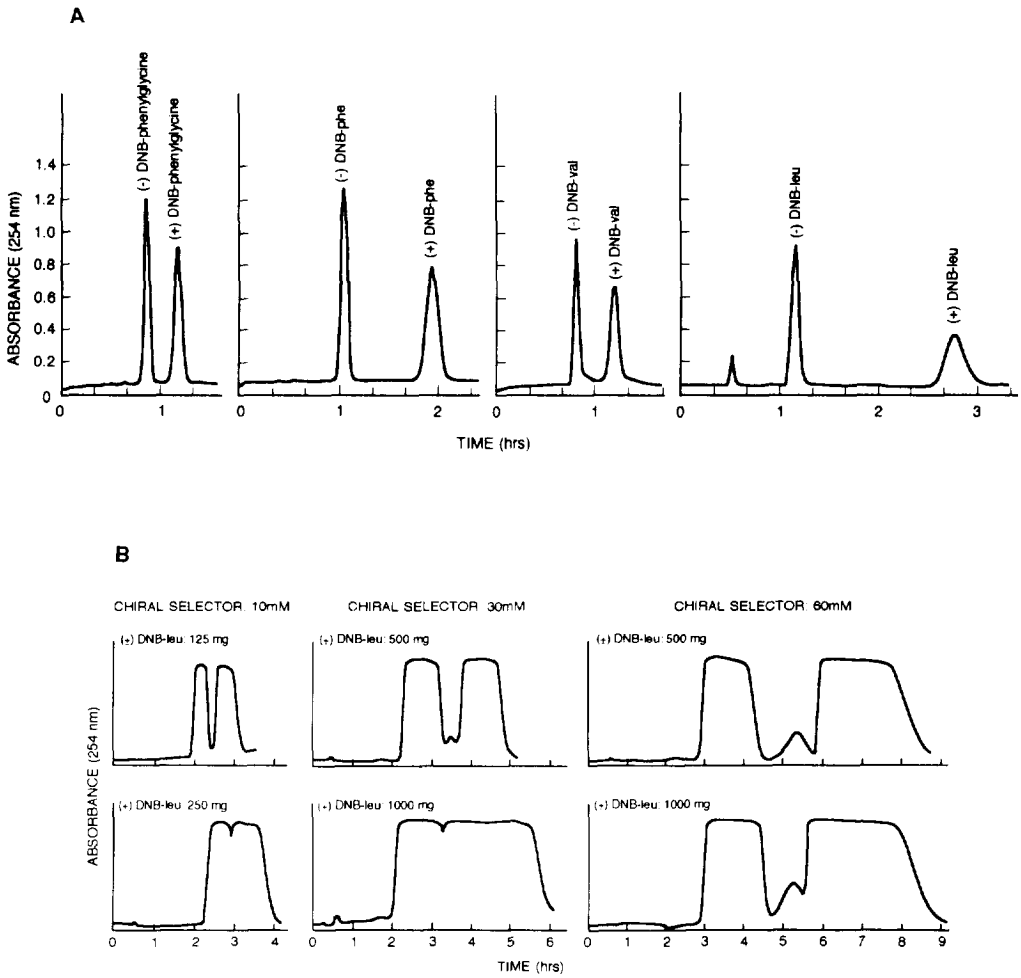

Fig. 2. Separations of (±)-DNB-amino acids by the standard CCC technique in analytical (A) and preparative (B) scales. Experimental conditions: apparatus: multilayer coil high-speed CCC centrifuge with a semipreparative column of 1.6 mm I.D. and 330 ml capacity; solvent system: (A) hexane–ethyl acetate–methanol–10 m*M* HCl (8:2:5:5), N-dodecanoyl-L-proline-3,5-dimethylanilide (2 g) was added to the organic stationary phase (200 ml) as a chiral selector (CS), (B) hexane–ethyl acetate–methanol–10 m*M* HCl (6:4:5:5) where the organic stationary phase containing CS at 10 to 60 m*M* as indicated. Samples: (A) from left to right: (±)-DNB-phenylglycine, (±)-DNB-phenylalanine, (±)-DNB-valine and (±)-DNB-leucine, 5–10 mg of each dissolved in 5 ml of solvent consisting of equal volumes of each phase; (B) (±)-DNB-leucine, 125–1000 mg dissolved in 10–45 ml of solvent. Flow-rate: 3.3 ml/min in the head-to-tail elution mode; revolution: 800 rpm; analysis of fractions: optical rotation and CD; stationary phase retention: 65% of the total column capacity.

indicate that the sample loading capacity is largely determined by the concentration (or net amount) of the chiral selector in the stationary phase (Fig. 2B): The higher the concentration of the chiral selector, the greater the peak resolution. The maximum sample size of 1 g was completely resolved in 9 h. Further increasing the sample size to 2 g, however, resulted in extensive carryover of the stationary phase apparently due to the formation of solid precipitate in the column. This problem was greatly alleviated by the pH-zone-refining CCC technique which allows use of more polar solvent systems.

pH-Zone-refining CCC [13–16] is a powerful preparative technique that is comparable to displacement chromatography [18] and isotacho-

Table 1
Separation factors of enantiomers of DNB-amino acids

Sample	R[a]	$D_{(\pm)}$[b]	$D_{(-)}$[c]	$D_{(+)}$[d]	α[e]
DNB-Phenylglycine	C_6H_5	0.17	0.19	0.41	2.13
DNB-Phenylalanine	$CH_2C_6H_5$	0.20	0.31	0.72	2.33
DNB-Valine	$CH(CH_3)_2$	0.18	0.19	0.43	2.27
DNB-Leucine	$CH_2CH(CH_3)_2$	0.30	0.37	1.36	3.43

[a]R = Side chain.
[b]$D_{(\pm)}$ = Distribution ratio (analyte concentration in the stationary phase divided by that in the mobile phase measured before the chiral selector was added to the solvent system).
[c]$D_{(-)}$ = Distribution ratio of (−)-enantiomer.
[d]$D_{(+)}$ = distribution ratio of (+)-enantiomer.
[e]α = Separation factor.

phoresis [19]. It yields a succession of highly concentrated rectangular solute peaks with minimum overlap where impurities are concentrated at the peak boundaries. Recently, the technique has been successfully applied to separations of wide varieties of compounds including amino acid derivatives [20,21], oligopeptide derivatives [22], a variety of hydroxyxanthene dyes [23–27], alkaloids [28], indole auxins [14], structural [29] and geometrical [30] isomers, etc.

In the present study this technique was used to resolve (±)-DNB-leucine using a binary two-phase solvent system composed of methyl *tert.*-butyl ether and water where a retainer acid (trifluoroacetic acid) and the chiral selector (same as that used in the standard CCC technique) were added to the organic stationary phase and an eluent base (ammonia) to the aqueous mobile phase.

Fig. 3 shows a typical chromatogram obtained by pH-zone-refining CCC, where two gram of (±)-DNB-leucine was eluted in a single rectangular UV peak in about 3 h. The pH of each fraction (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 while the narrow zone boundary contained both isomers and an impurity (see the upper diagram). This mixing zone is estimated to be no more than 5% of each peak.

In the absence of a complexing agent, as reported elsewhere [14,16], the separation of the analytes and their elution order in pH-zone-refining CCC are based on the zone pH which is expressed by the following equation

$$pH_z = pK_a + \log\left(\frac{K_D}{D_r} - 1\right) \quad (1)$$

where K_D is the partition ratio of the neutral analyte $\{[AH]_{org}/[AH]_{aq}\}$, and D_r is the distribution ratio for the retainer acid or analytes (formerly called apparent partition coefficient or K_r). In the present method, however, the organic phase contains the chiral selector CS that binds to AH with a formation constant, $K = [AHCS]_{org}/[AH]_{org}[CS]_{org}$. Consequently, Eq. 1 must be modified to

$$pH_z = pK_a + \log\left\{\frac{K_D}{D_r}\cdot(1 + [CS]_{org}K) - 1\right\} \quad (2)$$

provided that CS and its complexes are insoluble in the aqueous phase ($D_{CS} > 100$ for the present solvent system). Since both pK_a and K_D values of the two enantiomers are identical and D_r is common, the separation of the two pH zones must be derived from the difference in term $[CS]_{org}K$ for the two isomers. Eq. 2 clearly indicates that resolution of two enantiomers is improved by increasing CS concentration and/or

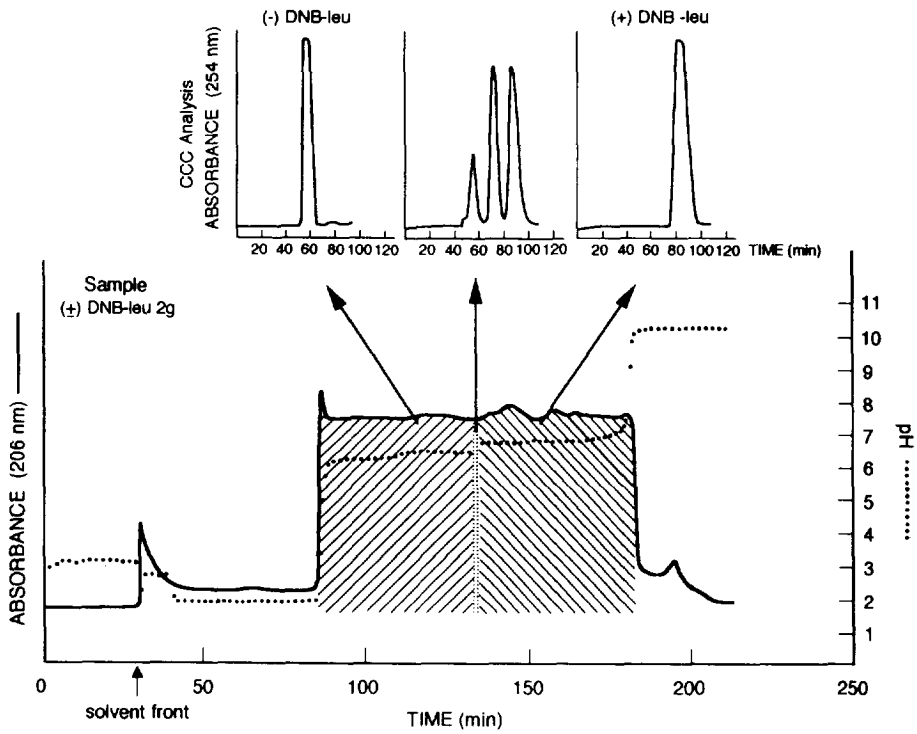

Fig. 3. Separation of (±)-DNB-leucine by pH-zone-refining CCC. Experimental conditions for pH-zone-refining CCC: Apparatus as in Fig. 2; solvent system: methyl *tert.*-butyl ether–water; stationary phase: upper organic phase to which TFA (40 m*M*) and CS (40 m*M*) were added; mobile phase: lower aqueous phase to which aqueous ammonia was added at 20 m*M*; sample: (±) DNB-leucine 2 g; flow-rate: 3.3 ml/min; revolution: 800 rpm; analysis: chirality by analytical high-speed CCC and pH by a portable pH meter. Experimental conditions for the analytical CCC as in Fig. 2A. Note that the analysis of the fraction from the mixing zone (middle chromatogram) shows three peaks corresponding to (−)-DNB-leucine, impurity and (+)-DNB-leucine from left to right.

the formation constant, K, for one of the isomers relative to the other.

Compared with the standard CCC technique described earlier, the pH-zone-refining CCC technique allows separation of larger amounts in shorter times. In addition, the method uses relatively polar solvent systems which can hold the chiral selector for much longer reducing contamination in the purified fractions. In both techniques, however, leakage of the chiral selector into the eluate can be completely eliminated by filling the outlet of the column with a suitable amount of CS-free stationary phase so that any chiral selector in the flowing mobile phase is retained.

Although our studies only show chiral separations of (±)-DNB-amino acids, it is obvious that the method may be extended to the separation of other racemic mixtures by choosing appropriate chiral selectors.

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