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CENTRIFUGAL COUNTER-CURRENT PARTITION CHROMATOGRAPHY WITH HELICAL COIL ROTOR

SIMPLIFIED COUNTER-CURRENT CHROMATOGRAPHY WITH A ROTATING FACE-SEAL

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SUMMARY

A centrifugal counter-current partition chromatograph has been developed and tested in order to simplify earlier counter-current chromatographic (CCC) procedures. It includes a helical coil rotor and a rotating face-seal. The rotor is designed to be adapted in an ordinary laboratory centrifuge for toroidal coil CCC. Twisting between the inlet and outlet tubing is avoided by using the rotating seal in the rotor. The seal is placed between the rotor, on which helical coils are mounted, and a newly designed centrifuge lid.

This chromatographic rotor, rotating simply around its own axis, has simplified a previous CCC device in which a coil planet mechanism is used to avoid tube twisting whilst retaining the capability for chromatographic separations. Results for separations of nystatin, dinitrophenylamino acids and Poly I:C were comparable to those obtained by liquid chromatography and the previous CCC procedure.

INTRODUCTION

Counter-current chromatography (CCC), proposed by Ito and co-workers[1,2], is a liquid–liquid partition technique performed with the aid of a centrifugal force field. In CCC, the use of solid supports is eliminated and a high partition efficiency comparable to that in liquid chromatography can be obtained.

The instrumentation for this technique is now developing in two ways: (a) CCC with Ito and co-workers' "seal-free flow-through coil planet centrifuge" and (b) centrifugal partition chromatography (CPC) with a centrifuge employing the Murayama *et al.*'s "microcell cartridges together with rotary seal joints"[3]. However, the fundamental principle, experimental methodology and fields of application of these two

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types of chromatography are almost identical. Owing to the simple rotation of a rotor, the CPC device is more easily constructed for commercial applications.

Disc-shaped toroidal coil rotors[4,5] are the simplest form in Ito and co-workers' CCC. The rotor consists of helically wound tubing mounted circumferentially around a horizontal rotating disc. It is easily manufactured and more easily accepted by users than other toroidal rotors and conventional coil planet centrifuges. Also, these rotors are simpler than those in CPC.

However, Ito and co-workers' seal-free flow-through CCC with a coil planet mechanism, even with the simplest rotors, have complicated mechanical structures and running noise, which have limited its further use.

In order to simplify the structure of Ito and co-workers' CCC device, we have developed a helical coil rotor by using a rotating face-seal for centrifugal counter-current partition chromatography (CCPC). This rotor for CCPC can also be simply applied in an ordinary laboratory centrifuge.

In this paper, the construction of this rotor and the operation of CCPC are described. Some examples of separations of nystatin, dinitrophenyl(DNP)-amino acids and Poly I:C are also demonstrated. The distributions of components by CCPC are compared with the results of similar separations obtained by other workers[6,7].

PRINCIPLE

Similarly to the description by Ito and co-workers[1,2], a coiled tube placed horizontally in a centrifugal field is first filled with one phase of an equilibrated two-phase solvent system; when the other phase is introduced into the coil from the inlet, it percolates through the first phase, leaving a segment of the first phase in each coil unit (Fig. 1). If the mobile phase is pumped into the coil continuously, then it only

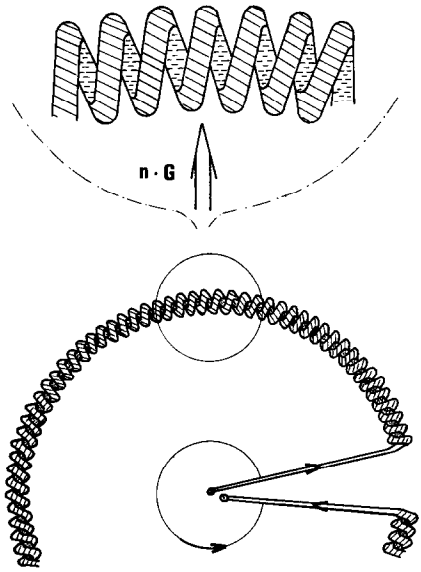

Fig. 1. Two immiscible liquids in a centrifugal field. $n{\cdot}G = n$ times the acceleration of gravity.

displaces itself, leaving the first phase stationary in each coil unit. When a sample consisting of several components is injected at the inlet and transported by the mobile phase, the sample is subjected to a partition process between the two phases and separated according to the relative partition coefficients of the components. Because hundreds of even thousands of turns of the coil can easily be produced, a high efficiency of partition chromatography can be expected.

Separation experiments with disc-type toroidal coil rotors[4,5] showed that the simple rotation of the rotor around its own axis could provide a counter-current partition process. In other words, the coil planet motion applied in Ito and co-workers' CCC device is not essential for the principle of CCC.

The fluid dynamic properties of a helical coil placed in a centrifugal force field are used to build segments in each turn of the coil as shown in Fig. 1, irrespective of whether the centrifugal force field is changing periodically, as in coil planet centrifuges[1,2], or is constant as in disc-type toroidal rotors[4,5] and in our helical coil rotor for CCPC. Hence partition chromatographic separations also occur in these simply rotating rotors. Therefore, a helical coil rotor for CCPC was designed and built in which the disc rotor undergoes simple rotation. In our experiments the formation of segments between two phases was demonstrated by adding a red dye to one phase for easy visual examination.

APPARATUS

The CCPC system developed in our laboratory is based on a centrifuge. Its helical coil rotor has a similar shape to other rotors[4,5] and is mounted in the bowl of a Model HSC 20R high-speed refrigerated centrifuge (Tumen Centrifuge Factory, Tumen City, Jilin Province, China). This rotor is connected to the hub of the drive shaft just like other rotors in this centrifuge, without any modification to the centrifuge except for a new lid that has holes to instal the rotating seal unit. The lid can be made of Perspex in order to be able to examine the rotor while it is rotating (Fig. 2).

The rotor includes a CCC separation part, consisting of the helical coil, and a rotating face-seal unit. The former is the chromatographic column and the latter is used to avoid twisting between the inlet and outlet tubing. The rotating face-seal unit permits liquid to be pumped in and out of the rotating helical coil during rotation of the rotor. The rotor diameter is 330 mm.

The helical coil is fine tubing wound on a flexible former. This coil can be mounted on a disc with several turns circumferentially; there are eight clamps for locating it. The disc also can be provided with a shoulder to support the helical coil radially. The tubing used in CCPC is usually made of PTFE of I.D. ranging from less than 1 mm to several millimetres.

The rotating face-seal unit consists of a rotating seal member, a stationary seal member, a preloading unit and a bearing unit (Fig. 3).

The rotating seal member (lower seal) is a disc made of stainless steel or other suitable hard material, carefully machined to a high degree of flatness and with a mirror finish on the centre of the rotor.

The stationary seal member (upper seal) is made of PTFE also machined to a fine flat finish. It is mounted on the lower surface of a spring holder. Both the rotating seal member and stationary seal member have an inlet and outlet. The liquid phase is

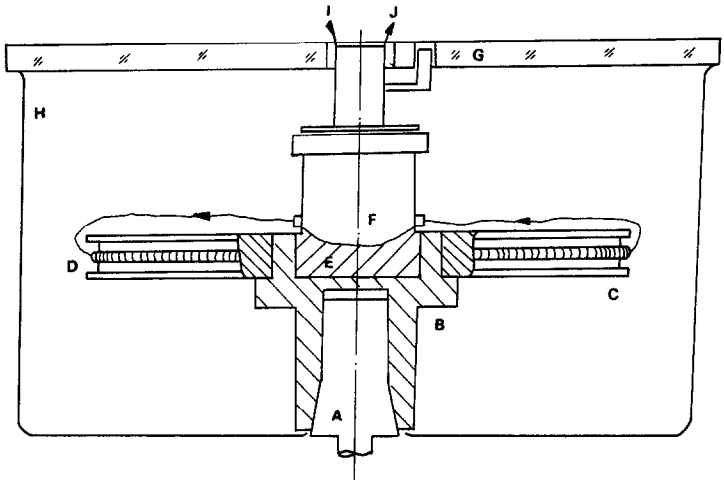

Fig. 2. Diagram of the rotor for CCPC in a centrifuge. A = Hub of centrifuge; B = body; C = disc; D = PTFE coil tubing; E = core; F = rotating face-seal unit; G = lid; H = centrifuge bowl; I = inlet tubing; J = outlet tubing.

delivered through these two seal members via two holes, one directly through the centre and another slightly offset. The stationary phase, the mobile phase and the sample are introduced into the helical coil through the central holes. A small circular groove on the surface of the PTFE seal collects the outlet from the offset hole in the stainless-steel seal for delivery to the detector.

The sealing surfaces were preloaded by a coil spring (not shown) located inside a spring holder. The preloading pressure can be regulated by a screw in a plug inside a housing. Between the housing and the sleeve of the upper part of the rotor is a ball bearing. The housing is connected to the centrifuge lid by an arm to prevent the stationary seal member from turning during rotation of the rotor.

The route of the liquid is as follows. The liquid is pumped into the central holes of the static and rotating seal member through the inlet tubing, passed through the helical coil and the offset holes of the rotating and static seal member, and is drained

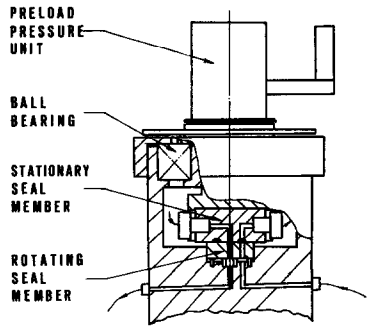

Fig. 3. Rotating face-seal unit.

from the outlet tubing to a detector. The helical coil and rotating seal member rotate together with the rotor, but the stationary seal member does not rotate, so the inlet and outlet tubes do not twist.

EXPERIMENTAL

The CCPC operating system is shown schematically in Fig. 4. A piston pump (Model YSB-2; Shanghai Instruments, Academia Sinica, China) was used to pump the phases into the helical coil.

The eluate from the outlet of the coil was continuously monitored with an LKB Uvicord S detector at a suitable wavelength and the absorbance was recorded with an LKB 2210 recorder. An LKB 2070 Ultrorac II fraction collector was used to collect the eluate.

The helical coil was prepared from a single piece of PTFE tubing of 0.6 mm I.D. (Shanghai Chemical Works, China) by winding it tightly onto a coil former of 3 mm diameter. The inlet and outlet tubing were also PTFE tubing (0.5 mm I.D.).

All organic solvents used were of analytical-reagent grade. Each two-phase solvent system was equilibrated at room temperature.

For testing the capability of CCPC, experiments were carried out with nystatin (supplied by the Beijing Institute for Control of Pharmaceuticals as a Chinese working standard), poly I:C (concentration 1 mg/ml, supplied by the 852005 group of our Institute) and the dinitrophenyl(DNP)-amino acids: DNP-L-aspartic acid, DNP-L-glutamic acid, DNP-L-alanine and DNP-L-leucine (Dongfeng Chemicals, Shanghai, China). The sample solutions were prepared by dissolution in the upper or lower phase of the respective solvent system and were stored in the dark at 4°C.

The separation was performed as follows: The column was filled with the stationary phase and the sample solution was introduced into the inlet of the column, then the mobile phase was pumped into the column while the rotor was running at a suitable speed.

In the experiments on the separation of nystatin, the upper layer of chloroform–methanol–borate buffer (pH 8.2) (2:4:3) was used as the stationary phase and the lower layer was pumped at a rate of 6 ml/h as the mobile phase. The nystatin

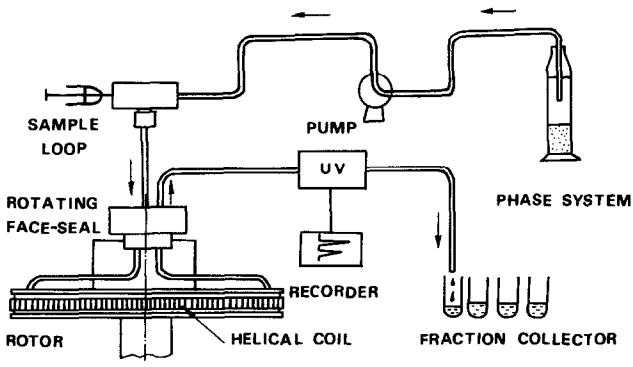

Fig. 4. Schematic view of CCPC operating system with a helical coil rotor.

sample solution was prepared by dissolving 10 mg of nystatin in 1 ml of the lower phase. The column was 10 m long with 710 helical turns. Its total capacity was about 2.8 ml. The rotor speed was 710 rpm.

In the separation of the mixture of four DNP-amino acids, the two-phase solvent system was composed of chloroform–glacial acetic acid–0.1 *M* hydrochloric acid (2:2:1). The upper layer was used as the mobile phase at a flow-rate of 1.8 ml/h. The rotor speed was 450 rpm. A 10-mg amount of each DNP-amino acid was dissolved in 2 ml of the upper phase. The column was 47 m long with about 4000 helical turns.

In the separation of Poly I:C, the two-phase solvent system consisted of 5% Dextran T 500 (Pharmacia), 4% polyethylene glycol 6000 (produced in Japan, supplied by Foshan Chemicals, China), 10 m*M* sodium phosphate and 0.15 *M* sodium chloride (Beijing Chemicals, China). The lower phase was used as the stationary phase. The sample injection volume was 30–50 μl, the flow-rate was 0.6–1.2 ml/h using gradient elution, the rotor speed was 300–400 rpm and the columns of different lengths (19, 54 and 77 m) were used.

RESULTS AND DISCUSSION

Fig. 5 shows the chromatogram for nystatin obtained by CCPC. There are several peaks in addition to three main peaks in the upper layer. Fig. 6 shows the separation of nystatin obtained by high-performance liquid chromatography (HPLC) using a Perkin-Elmer III liquid chromatograph with an ODS-HCSIL-X-1 column (25 cm × 0.26 cm I.D.) with methanol–distilled water (68:32) as the mobile phase and

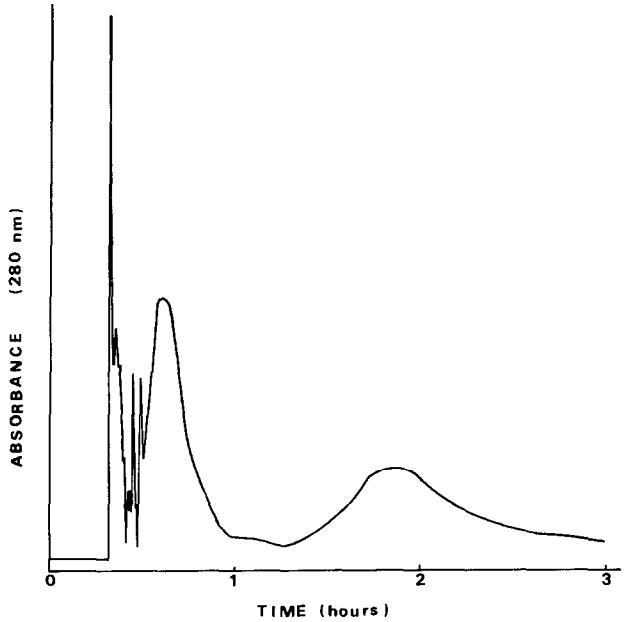

Fig. 5. Chromatogram of nystatin obtained by CCPC. Solvent system: chloroform–methanol–borate buffer (2:4:3); mobile phase, lower phase; flow-rate, 6 ml/h; rotor speed, 710 rpm; sample dose (volume), 50 μg (5 μl); column pressure, 8.0 kg/cm^2; tubing length, 10 m.

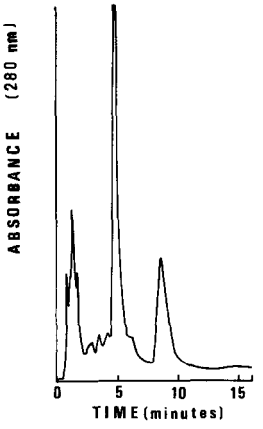

Fig. 6. HPLC results for nystatin. Mobile phase, methanol–water (68:32); sample dose (volume), 30 μg (3 μl).

detection at 280 nm. The results in Figs. 5 and 6 are nearly same: CCPC gives a better resolution than HPLC. Our results confirm those reported using HPLC[8].

Fig. 7 shows the chromatogram of a mixture of the four DNP-amino acids by CCPC and Fig. 8 shows the HPLC results for the same mixture obtained using a Hewlett-Packard HP 1090 A liquid chromatograph with a Hypersil ODS (3 μm) column (60 × 4.6 mm I.D.). The results indicate that the CCPC has a high separating efficiency, comparable to that of Ito and co-workers CCC method[1] and HPLC. The samples that we used were not chromatographic reagents, and therefore there are some impurity peaks in addition to the main peaks.

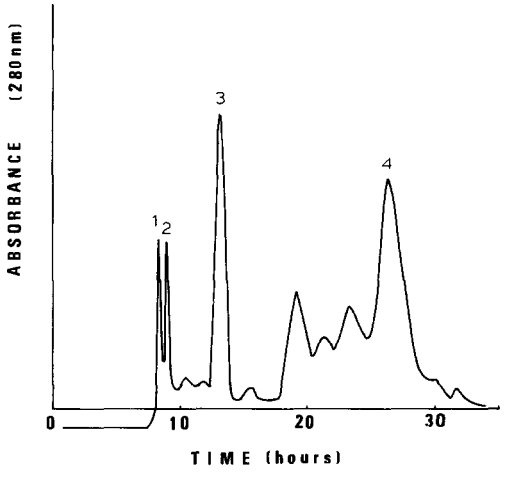

Fig. 7. Chromatogram of DNP-amino acids obtained by CCPC. Solvent system, chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1); mobile phase, upper aqueous phase; flow-rate, 1.8 ml/h; rotor speed, 450 rpm; sample dose (volume), 100 μg (5 μl); column pressure, 11 kg/cm^2; tubing length 47 m. Peaks: 1 = α-DNP-L-aspartic acid; 2 = α-DNP-L-glutamic acid; 3 = α-DNP-L-alanine; 4 = α-DNP-L-leucine.

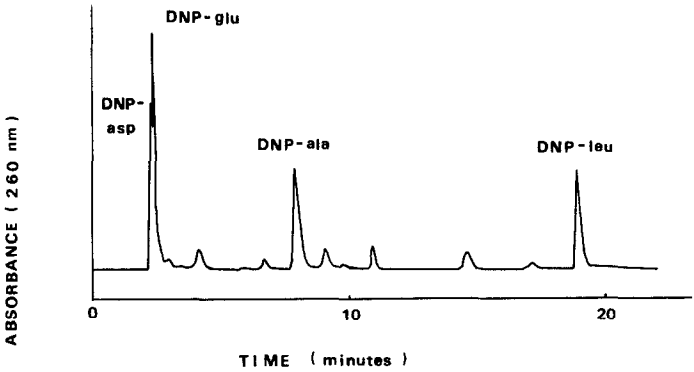

Fig. 8. HPLC results for DNP-amino acids. Mobile phase, (A) 0.01 *M* acetic acid–(B) ethanol. Gradient elution: at 5 min 15%, 7 min 20%, 10 min 25%, 15 min 30%,17 min 33% and 20 min 35% B. Flow-rate, 16.8 ml/h; sample volume, 1–3 μl.

Fig. 9 shows the chromatogram of Poly I:C obtained by CCPC. There are four main peaks (A–D) with some split peaks, indicating different components of polynucleotides. Their sedimentation coefficients are 12S, 7.0S, 6.35S and 3.8S, respectively, measured with a Hitachi 282 ultracentrifuge (RA 72 TC rotor, 53 000 rpm, UV detector). Different coil lengths (19, 54 and 77 m) and flow-rates (0.6 and 1.2 ml/h) gave similar results, better than those in earlier work[7]. The details will be published elsewhere. The liquid chromatogram of Poly I:C contained three peaks, one main peak and two much smaller peaks. The CCPC results are much better than those obtained by LC. The chromatograms of DNP-amino acids and Poly I:C show that the capability of CCPC is comparable to that of Ito and co-workers' CCC method

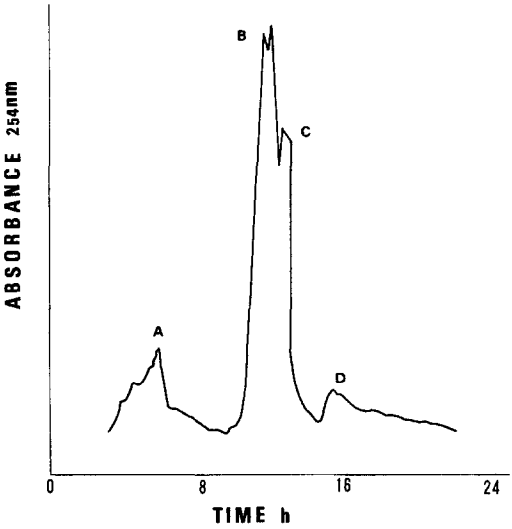

Fig. 9. Chromatogram of Poly I:C obtained by CCPC. Solvent system, 5% Dextran T500–4% polyethylene glycol 6000–0.15 *M* sodium chloride; mobile phase, upper aqueous phase; flow-rate, 0.6 ml/h; rotor speed, 350 rpm; sample volume, 50 μl; column pressure, 3 kg/cm^2; tubing length, 19 m.

and the chromatograms of DNP-amino acids and nystatin show that the separation results of CCPC are comparable to those of HPLC, although the separation time is much longer (more than ten times) with the former method.

The use of the rotating seal unit simplified the previous CCC device of Ito and co-workers. However, strictly the rotating seal is a potential source of leakage and contamination of hazardous materials, although similar structures have been successfully used since the 1960s in zonal centrifugation, continuous-flow centrifugation and, more recently, elutriation centrifugation and centrifugal partition chromatography. So far, about 7000 h of accumulated running time of the CCPC system over more than 3 years have been recorded using these seals, and their reliability is fairly good.

CCPC has inherited the merits of Ito and co-workers' CCC method of the elimination of the solid supports, high sample recovery, high purity of fractions and retention of biological activity, and is cheaper than HPLC. In addition, because the rotor in CCPC is a simple rotation device as in all centrifuge rotors, without complicated planetary motion, this CCPC method can be used in an ordinary centrifuge by adding a CCPC rotor, and can also be utilized in other simply designed centrifuges. In both instances, refrigeration to maintain biological materials at lower temperatures is easily provided, whereas it may be a problem in current CCC devices according to Ito and co-workers.

Compared with our CCPC, some CCC devices based on Ito and co-workers principle has one additional operating parameter, *i.e.*, revolution speed, which can be chosen to meet various needs for some samples. Therefore, the latter system may give better separation results for certain samples than the present CCPC method. In addition, current CCC devices according to Ito and co-workers with coil planet mechanism have a flow-through design to avoid twisting between the inlet and the outlet, whereas a rotating seal is used in our CCPC system. To assemble this rotating seal, certain skill is required, and the seal has a limited lifetime (about 500–1000 h).

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