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IMPROVED CROSS-AXIS SYNCHRONOUS FLOW-THROUGH COIL PLANET CENTRIFUGE FOR PERFORMING COUNTER-CURRENT CHROMATOGRAPHY

II. STUDIES ON RETENTION OF STATIONARY PHASE IN SHORT COILS AND PREPARATIVE SEPARATIONS IN MULTILAYER COILS

MOLINA BHATNAGAR, HISAO OKA* and YOICHIRO ITO*
Laboratory of Technical Development, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, Room 5D12, 9000 Rockville Pike, Bethesda, MD 20892 (U.S.A.)
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SUMMARY

Performance of the apparatus was evaluated in terms of stationary phase retention, partition efficiency and sample loading capacity. Preliminary studies with short coils revealed high retention of the stationary phase under a proper combination of the head–tail elution and planetary motion. Preparative capability of the apparatus was successfully demonstrated on efficient multigram separations of 2,4-dinitrophenyl amino acids, indole auxins, and bacitracin in a pair of large multilayer coils with a total capacity of 1.5 l.

INTRODUCTION

As described in Part I[1], the present design of the cross-axis synchronous flow-through coil planet centrifuge (X-axis CPC) generates a characteristic centrifugal force field which will permit high retention of the stationary phase in the coiled column.

In this paper, perfomance of the present apparatus was studied in terms of stationary phase retention, partition efficiency, sample loading capacity, etc. A series of systematic studies was conducted to measure retention of the stationary phase in short single-layer coils mounted on two column holders with different hub diameters. Using the optimal experimental conditions determined by the preliminary studies, the preparative capability of the present apparatus was demonstrated on gram-quantity separations of various biological samples in a pair of large multilayer coils connected in series with a total capacity of 1.5 l.

* Visiting scientist from Aichi Prefectural Institute of Public Health, Nagoya, Japan.

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EXPERIMENTAL

Apparatus

The design of the apparatus used in the present studies is described in detail in Part I[1]. Briefly, the present X-axis CPC symmetrically holds a pair of multilayer coils on the rotaty frame 10 cm from the center of the holder shaft (Fig. 1). This lateral position of the columns allows for the use of larger multilayer coils and also yields higher retention of the stationary phase.

Separation columns

Present studies were performed with two different types of coiled columns: single-layer short coils for preliminary phase retention studies and long multilayer coils for preparative separation studies.

Short coils were prepared with a 5 m × 2.6 mm I.D., and 28 ml capacity PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) by manually winding a single layer onto the holder hub of either 10 or 20 cm diameter. The column was mounted at a lateral position 10 cm left from the center of the holder shaft. A counterweight was mounted on the opposite side of the rotary frame symmetrically in the lateral position. Each coil was securely affixed onto the holder hub with fiberglass reinforced adhesive tape. Each end of the coil was connected to a flow tube, measuring *ca.* 1 m × 0.85 mm I.D. These flow tubes were directed to the outside of the centrifuge as described in Part I[1].

Each multilayer coil was prepared from one piece of 2.6 mm I.D. PTFE tubing by winding it in a spool-type fashion onto the holder of 15 cm diameter, thus making multiple layers between a pair of flanges spaced 5 cm apart. The column consisted of 15 layers of coiled tubing with a capacity of about 750 ml. The β value varied from 0.75 at the internal terminal to 1.15 at the external terminal. As described in Part I[1], β is the

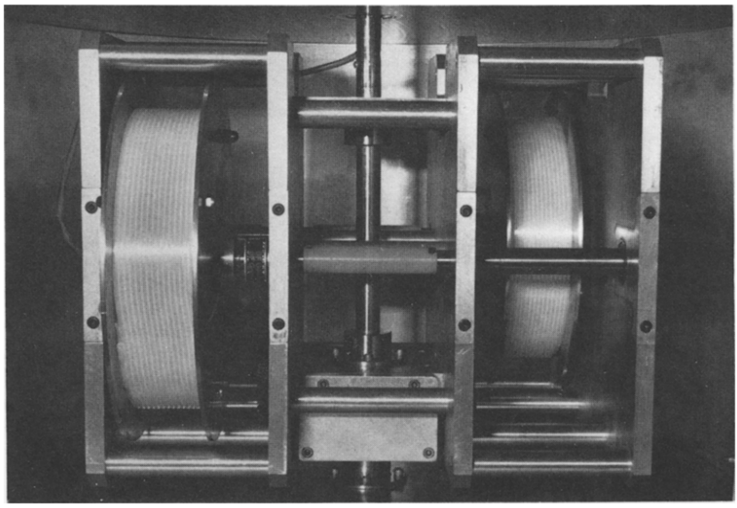


Fig. 1. The present apparatus equipped with a pair of large multilayer coil separation columns in the lateral position.

ratio of the radius of rotation (distance from the central axis of the holder to the coil) to the radius of revolution (distance from the central axis of the centrifuge to the axis of the holder). A similar second multilayer coil was laterally placed in a symmetrical position, on the opposite side of the rotary frame (thus making the total column capacity about 1.5 l). These two columns were connected in series with a flow tube on the rotary frame in such a way that the external terminal of the first column and the internal terminal of the second column were joined. In this way, mechanical balance of the centrifuge system was ensured and the two columns had identical elution modes. Fig. 1 shows the present apparatus equipped with a pair of multilayer coils in the lateral position of the holder shaft. This column location is of great importance as it allows for the utilization of large columns with greater volume capacities and improved stationary phase retention.

Reagents

Organic solvents used for the preparation of the two-phase solvent systems include *n*-hexane, ethyl acetate, chloroform, *n*-butanol, *sec.*-butanol, ethanol, methanol, hydrochloric acid, and acetic acid. Among these, acetic acid and ethanol were of reagent grade and obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.); and Midwest Solvents Co. (Pekin, IL, U.S.A.); respectively, while hydrochloric acid (1 *M*), and all test samples such as 2,4-dinitrophenyl amino acids (DNP-L-leucine, DNP-L-proline, DNP-β-alanine, DNP-DL-glutamic acid, diDNP-L-cystine, DNP-Δ-L-ornithine, DNP-L-aspartic acid, and DNP-L-alanine), indole auxins (indole-3-acetamide, indole-3-acetic acid, indole-3-butyric acid), and bacitracin were obtained from Sigma (St. Louis, MO, U.S.A.). All other solvents were glass-distilled chromatographic grade and purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Preparation of two-phase solvent systems and sample solutions

Using the solvents mentioned above, the following volatile two-phase solvent systems were prepared for preliminary studies on retention of the stationary phase: *n*-hexane–water; *n*-hexane–methanol; *n*-hexane–ethyl acetate–methanol–water (1:1:1:1); ethyl acetate–water; ethyl acetate–acetic acid–water (4:1:4); chloroform–water; chloroform–acetic acid–water (2:2:1); *n*-butanol–water; *n*-butanol–acetic acid–water (4:1:5); and *sec.*-butanol–water. The solvent systems of chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1); *n*-hexane–ethyl acetate–methanol–water (3:7:5:5); and chloroform–95% ethanol–water (5:4:3) were prepared for the preparative separations with multilayer coils. Each solvent system was carefully equilibrated in a separatory funnel at room temperature by vigorously shaking and repeatedly degassing the mixture and by allowing the mixture to completely separate before its use.

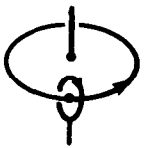
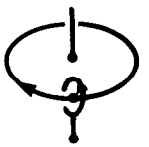
Sample solutions for DNP amino acid separations were prepared in the following manner: two sets of sample mixtures were prepared, one for the lower phase elution and the other for the upper phase elution, both with the same solvent system of chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1). The sample for the lower phase elution consisted of DNP-Leu (400 mg), DNP-Pro (800 mg), DNP-β-Ala (800 mg), diDNP-$(Cys)_2$ (400 mg), and DNP-Glu (1600 mg) dissolved in equal volumes of upper and lower phases totaling 100 ml of solvent. The second set of the sample for the upper phase elution consisted of DNP-Orn (400 mg), DNP-Asp (800 mg), DNP-Glu

TABLE I

SUMMARY OF EXPERIMENTAL CONDITIONS FOR PREPARATIVE CCC

A = Chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1); B = *n*-hexane–ethyl acetate–methanol–water (3:7:5:5); C = chloroform–95% ethanol–water (5:4:3).

Experiment No.	*Sample*	*Sample volume (ml)*	*Settling time (s)*	*Solvent system*	*Mobile phase*	*Flow-rate (ml/h)*	*Planetary* motion*	*Elution mode*	*Revolution speed (rpm)*
1	DNP amino acid mixture DNP-L-leucine 400 mg DNP-L-proline 800 mg DNP-β-alanine 800 mg diDNP-L-cystine 400 mg DNP-DL-glutamic acid 1600 mg	100	37	A	Lower phase	120	P_I	Head-to-tail	600
2	DNP amino acid mixture DNP-L-ornithine 400 mg DNP-L-aspartic acid 800 mg DNP-DL-glutamic acid 800 mg diDNP-L-cystine 400 mg DNP-L-alanine 1600 mg	100	37	A	Upper phase	120	P_I	Tail-to-head	600
3	Indole auxins Indole-3-acetamide 1 g Indole-3-acetic acid 1 g Indole-3-butyric acid 1 g	140	36	B	Lower phase	120	P_I	Head-to-tail	600
4	Bacitracin 5 g	120	100	C	Lower phase	120	P_I	Head-to-tail	600

* Planetary motion P_I:  ; P_{II}:  Large circles indicate revolution around the central axis of the centrifuge and small circles rotation around the holder axis.

(800 mg), diDNP-$(Cys)_2$ (400 mg), and DNP-Ala (1600 mg). The 4-g sample was similary dissolved in 100 ml of solvent, again using equal volumes of upper and lower phases. Indole-3-acetamide, indole-3-acetic acid, and indole-3-butyric acid, each 1 g, were dissolved in 140 ml solvent mixture consisting of equal volumes of the two phases. Bacitracin (5 g) was dissolved in 120 ml of solvent consisting of 30 ml water, 40 ml ethanol (95%), and 50 ml chloroform which were added in the above order and amounts.

Measurement of settling time of sample solution

The settling time of the sample solutions used in the preparative separations were measured by using the following procedures as described elsewhere[2]. First, the two-phase solvent system was equilibrated with the desired sample. Then, 2 ml of each phase were delivered into a 5-ml capacity graduated cylinder equipped with a glass stopper. The cylinder was gently shaken by carefully turning it upside down and returning it to its upright position five times. The cylinder was then placed on a horizontal surface, and the time required for the solvent mixture to form two clear layers was measured with a stopwatch. The measurement of the settling time was repeated several times for each sample solution to obtain the mean value. The settling times thus obtained are listed in Table I.

Procedures for preliminary studies on stationary phase retention in short coils

For each experiment, the short coil was first entirely filled with the stationary phase. Then the apparatus was run at the desired revolutional speed while the mobile phase was continuously eluted through the coil at a flow-rate of about 120 ml/h. The effluent from the outlet was collected in a 25-ml capacity graduated cylinder. After 20 ml were eluted, results were recorded by simply observing the amount of upper and lower phases comprising the total volume of the effluent. The apparatus was then flushed out by allowing nitrogen gas to push out the material in the column while rotating the apparatus at 100–200 rpm in the tail-to-head elution mode to facilitate the process. The experiments were performed by varying experimental conditions such as revolutional speeds (200, 400, 600, 800 rpm), planetary motions (P_I and P_{II}), elution modes (head-to-tail or tail-to-head), and the use of upper and lower phases as the mobile phase.

Construction of phase distribution diagram

From the volume of the stationary phase eluted from the column (V_s), the percentage retention of the stationary phase relative to the total column capacity was computed according to the expression, $100(V_c + V_f - V_s)/V_c$, where V_c and V_f are the total column capacity and the dead space volume in the flow tubes, respectively. Using these retention data, a set of phase distribution diagrams was prepared by plotting the percentage retention of the stationary phase against the applied revolutional speed in rpm. The retention curves obtained from the different elution modes of head–tail elution and planetary motion, but otherwise identical experimental conditions, were drawn in the same diagram to facilitate comparison. In order to distinguish the applied elution mode, each retention curve was drawn with a specific symbolic design as indicated in Table II.

TABLE II

FOUR DIFFERENT ELUTION MODES AT LATERAL COIL POSITION ($l = -10$ cm)

*Planetary motion**	*Head–tail elution mode*	*Combined elution mode**	*Symbolic designs in PDD***
P_I	Head-to-tail	P_I–H	○———○
	Tail-to-head	P_I–T	○- - - -○
P_{II}	Head-to-tail	P_{II}–H	△———△
	Tail-to-head	P_{II}–T	△- - - -△

* H = Head-to-tail; T = tail-to-head.
** PDD = Phase distribution diagram (Fig. 2).

Procedures for preparative separations with multilayer coils

Using the optimum conditions determined by the preliminary experiments with the short coils, preparative-scale counter-current chromatography (CCC) separations were performed with a pair of multilayer coils symmetrically mounted, one on each side of the centrifuge rotor, at the lateral positions on the holder shaft.

Each preparative separation was performed as follows: the entire column (the pair of multilayer coils connected in series) was first filled with the stationary phase. This was followed by the introduction of the sample solution through the sample port. Then, the apparatus was run at 600 rpm in planetary motion P_I while the mobile phase was pumped into the column at a constant flow-rate of 120 ml/h in the suitable head–tail elution mode. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 278 nm and fractionated with an LKB fraction collector to obtain approximately 15 ml of fraction in each tube (during a 7.5-min interval). An aliquot of fraction, 50 μl for the DNP amino acid and indole auxin separations and 0.5 ml for the bacitracin separation, was mixed with 3 ml methanol, and, using a Zeiss PM6 spectrophotometer, the absorbance of these samples was measured at suitable wavelengths of 430 nm for DNP amino acids, 280 nm for indole auxins, and 250 nm for bacitracin.

After each separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressured nitrogen gas combined with the slow rotation of the coil in the tail-to-head elution mode.

High-performance liquid chromatographic (HPLC) analysis of bacitracin

Both the original sample solution and the peak fraction of bacitracin obtained by the CCC separation were analyzed with a Shimadzu HPLC system consisting of a Model LC-6A pump, a manual injector kit, a Model SPD-6A detector, and a Model C-R5A recording data processor. HPLC analysis was performed on a Capcell Pak C_{18} column (15 × 0.46 cm I.D., type AG, Shiseido, Tokyo, Japan) by using a solvent composed of methanol and 0.04 *M* Na_2HPO_4 (pH 9.4) at a volume ratio of 62:38. At a flow-rate of 1 ml/min, the chromatogram was isocratically obtained by monitoring the absorbance at 234 nm.

RESULTS AND DISCUSSION

Model studies on retention in short coils

In the series of preliminary studies, ten different two-phase solvent systems were used to investigate the retention of the two solvent phases in the short coils mounted on a set of holders laterally located on the holder shaft. The results obtained clearly show that the degree of stationary phase retention is significantly affected by the direction of the planetary motion as well as the head–tail elution modes.

The results of the stationary phase retention studies are summarized in Fig. 2A. In this figure, each column consists of phase distribution diagrams obtained from the solvent systems labeled at the top of each column. These columns are arranged from left to right in the order of hydrophobicity of the major organic solvents (*i.e.*, *n*-hexane, ethyl acetate, chloroform, *n*-butanol, and *sec.*-butanol). The top two rows of the figure show retention of the lower phase obtained by the elution of the upper mobile phase, while the bottom two rows show the retention of the upper phase by the elution of the lower phase. Within each stationary phase group, the first row shows results obtained from a 10-cm holder diameter (or $\beta = 0.5$) and the second row shows results from a 20-cm holder diameter (or $\beta = 1.0$). Parameter β determines both the direction and magnitude of the centrifugal force field acting on various locations of the holder as described in Part I[1]. The two different elution modes are represented in Table II as the following: the solid line indicates the head-to-tail elution and the dotted or broken line the tail-to-head elution. The direction of the planetary motion is displayed as well: circles represent planetary motion P_I while triangles show the opposite planetary motion P_{II}.

Hydrophobic binary solvent systems, including *n*-hexane–water, ethyl acetate–water, and chloroform–water, show high stationary phase retention when the upper phase is eluted from tail to head (broken lines in the upper half of the figure) with planetary motion P_I or when the lower phase is eluted in the opposite elution mode of head-to-tail in planetary motion P_I (solid lines in the bottom half of the figure). These facts suggest that the upper phase tends to move toward the head and the lower phase toward the tail. The hydrophilic solvent systems such as *n*-butanol–acetic acid–water (4:1:5) and *sec.*-butanol–water, however, display an opposite hydrodynamic behavior. Planetary motion P_I, along with the head-to-tail elution of the upper mobile phase, brings about satisfactory retention levels (about 50%) for the lower phase. In observing upper phase retention levels, planetary motion P_{II} with the head-to-tail elution also produces similar retention levels of the stationary phase. The rest of the solvent systems with intermediate degrees of hydrophobicity generally show a hydrodynamic trend similar to that of the hydrophobic binary solvent systems with high retention levels.

The overall results of the above retention studies on the present apparatus indicated significant improvement of retention over those obtained from the central coil position in the original X-axis CPC[3,4]. Intermediate solvent systems used in the original apparatus with the central coil position show reversed hydrodynamic trends from those found in the present studies. In the present apparatus, intermediate solvent systems such as *n*-hexane–methanol, ethyl acetate–acetic acid–water (4:1:4), and *n*-butanol–water, display excellent retention at $\beta = 0.5$ or 1.0 under the proper head-to-tail elution mode combined with planetary motion P_I. Tremendous improve-

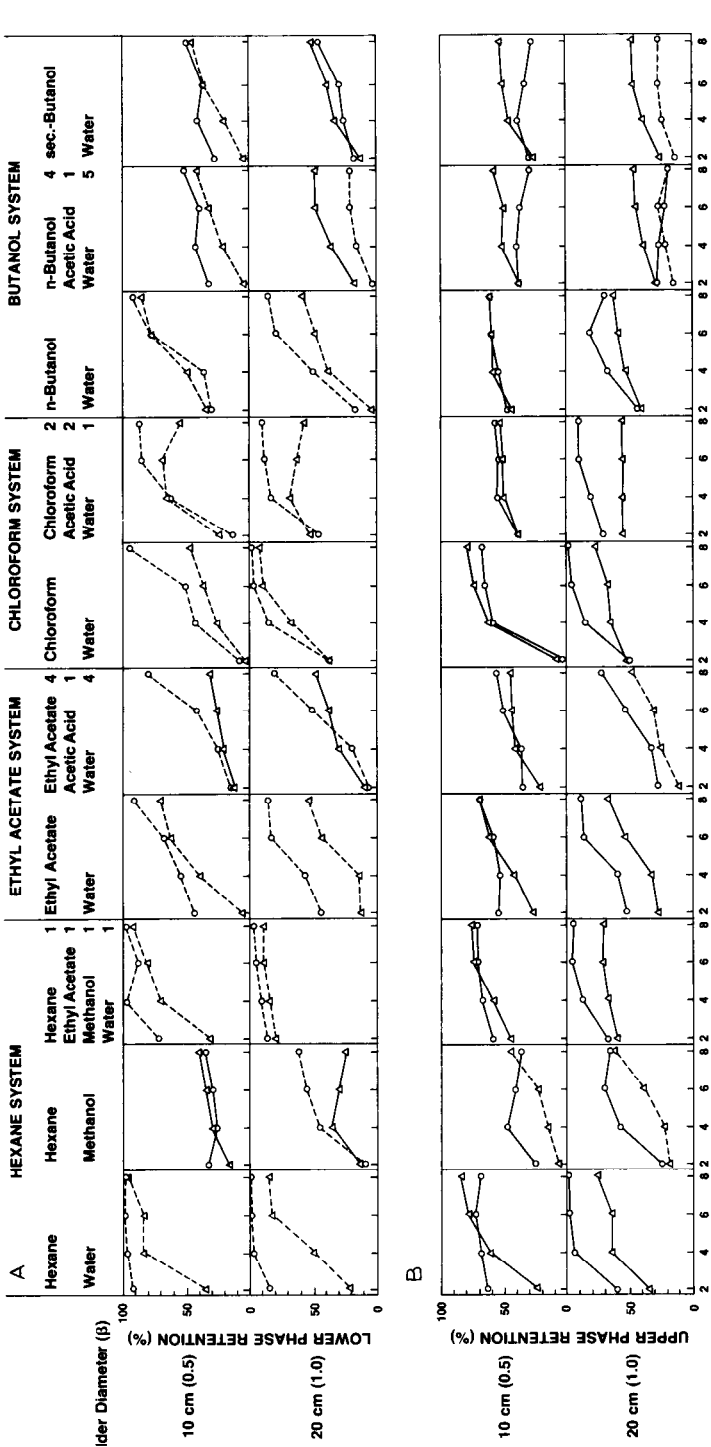


Fig. 2. Phase distribution diagrams obtained from the present X-axis CPC (type L–X, 10 cm revolution radius, $l = -10$ cm). See Table II for symbolic designs for phase distribution curves.

ment in retention with hydrophilic solvent systems was also observed. Retention values of *n*-butanol–acetic acid–water (4:1:5) and that of *sec.*-butanol–water reached approximately 50% at both β values. The above results clearly indicate that the present system, due to its lateral coil position, gives remarkable retention of the stationary phase in all the solvent systems examined, provided that the proper combination of planetary motion and head–tail elution mode is utilized. These results are consistent with the results of acceleration analysis described in Part I[1].

Preparative CCC with multilayer coils

Fig. 3A shows a chromatogram of the first set of DNP amino acid mixture with a solvent system composed of chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1) using the lower non-aqueous phase as the mobile phase. The separation was performed at a flow-rate of 120 ml/h at 600 rpm (P_I). The 4-g quantity of the sample mixture was efficiently separated within 24 h. DNP-glutamic acid was eluted as a skewed peak, apparently due to the non-linear isotherm caused by its high concentration in the sample solution. Fig. 3B shows a similar chromatogram of the second set of DNP amino acid mixture by eluting with the upper aqueous phase but otherwise identical experimental conditions. All peaks were well resolved and eluted out in 21 h. The retention of the stationary phase in these separations was 78.5% in the first experiment and 70.3% in the second experiment.

Fig. 4 shows a chromatogram of indole auxins with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5). The separation was performed by eluting the lower aqueous phase in the head-to-tail mode at a flow-rate of 120 ml/h at 600 rpm (P_I). Three components were well separated as discrete symmetrical peaks within 24 h. The partition efficiencies were computed according to the conventional gas chromatographic formula:

$$N = (4R/w)^2$$

where N denotes the partition efficiency expressed in terms of theoretical plate number, R, the retention time or volume of the peak maximum and w, the peak width expressed in the same unit as R. The present separation yielded high partition efficiencies ranging from 300 theoretical plates for the first peak to 700 theoretical plates for the second peak. The retention of the stationary phase was 70.8%.

The present method was applied to separation of commercial bacitracin sample using a solvent system composed of chloroform–95% ethanol–water (5:4:3). Bacitracin (5 g) was dissolved in 120 ml of solvent consisting of approximately equal volumes of the upper and the lower phases. The lower non-aqueous phase was used as the mobile phase and introduced through the head of the multilayer coil at a flow-rate of 120 ml/h at 600 rpm (P_I). In Fig. 5, a chromatogram of bacitracin thus obtained consists of three major peaks which include the second peak of bacitracin A as labeled.

Purity of the bacitracin A fraction obtained from the above separation was determined by a reversed-phase HPLC analysis. Fig. 6A shows the chromatogram obtained from the original sample solution which contains over 20 UV-absorbing peaks, including the main bacitracin A peak eluting at 11.8 min. Fig. 6B shows the chromatogram of the major peak fraction (fraction 85) obtained under the identical analytical condition which indicates high purity of bacitracin A with only few minor

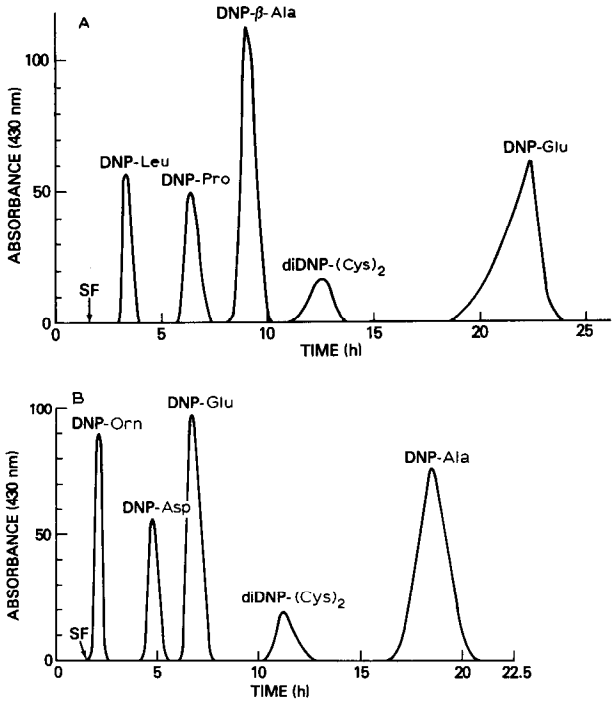


Fig. 3. Chromatograms of DNP amino acid mixture with chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1). (A) Lower phase mobile; (B) upper phase mobile. See Table I for experimental conditions.

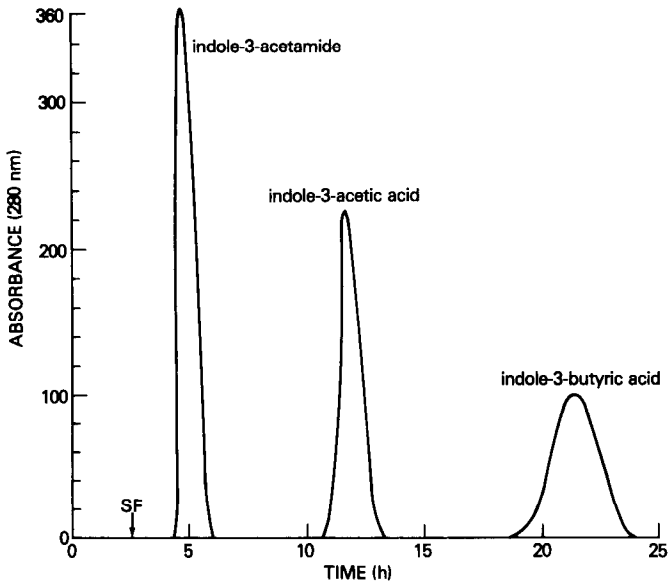


Fig. 4. Chromatogram of indole auxins with *n*-hexane–ethyl acetate–methanol–water (3:7:5:5). See Table I for experimental conditions.

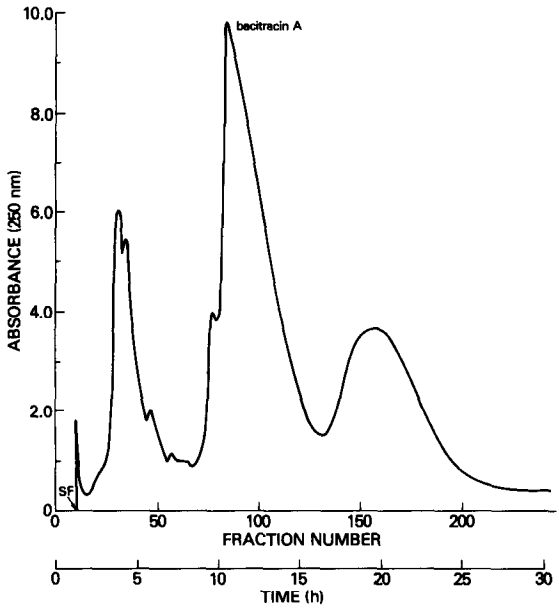


Fig. 5. Chromatogram of bacitracin with chloroform–95% ethanol–water (5:4:3). See Table I for experimental conditions.

peaks of impurity. The earlier eluted two peaks with retention times of 3.0 and 4.0 min were derived from chloroform and a possible impurity from ethanol used in the CCC separation.

In the above bacitracin separation, the retention of the stationary phase was found to be as high as 71%, which apparently produced excellent peak resolution in the present separation. Previously, it has been observed that heavy loading of sample solution with a long settling time of over 30 s tends to decrease retention of the

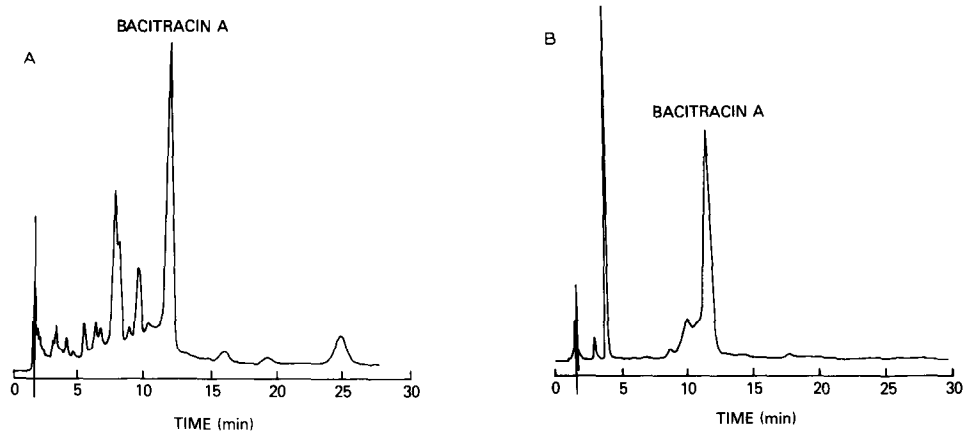


Fig. 6. HPLC analysis of bacitracin. (A) Chromatogram of original sample; (B) chromatogram of peak fraction 85 of bacitracin A. For experimental conditions, see Experimental section.

stationary phase resulting in poor peak resolution in the existing type J high-speed CCC instruments[2]. With the present apparatus, however, a 5-g quantity of bacitracin dissolved in 120 ml of solvent mixture with a long settling time of about 100 s was efficiently separated with a high retention value of over 70%. This unusual retention capability of the present X-axis CPC may be explained on the basis of the asymmetric lateral centrifugal force field resulted from lateral shift of the coil position as discussed in Part I[1].

The overall results of the above studies clearly indicate a great potential of the present apparatus for preparative-scale separations. The unique capability of the apparatus may be summarized as follows: (1) high retention of the stationary phase to yield excellent peak resolution; (2) high partition efficiency due to vigorous mixing; (3) universal choice of the two-phase solvent systems; (4) large column capacity and high sample loading capability; (5) stable balance of the centrifuge system without counterweight adjustment.

Because of these advantages, the present X-axis CPC will be extremely useful for preparative separations of a variety of natural and synthetic products in both research laboratories and industrial plants.

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