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IMPROVED HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPH WITH THREE MULTILAYER COILS CONNECTED IN SERIES

II. SEPARATION OF VARIOUS BIOLOGICAL SAMPLES WITH A SEMI-PREPARATIVE COLUMN

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SUMMARY

The semipreparative capability of the newly developed high-speed counter-current chromatograph equipped with a set of three multilayer coils has been demonstrated in separations of a variety of biological samples including triterpenoic acids, indole auxins, bacitracin, flavonoids and tetracycline derivatives, each with a suitable two-phase solvent system. The sample quantities ranging from 50 to 500 mg were efficiently separated within a few hours. The separation of tetracycline derivatives was remarkably improved by adding ammonium acetate to the solvent system.

INTRODUCTION

The newly developed high-speed counter-current chromatography (HSCCC) system eliminates the use of a counterweight by integrating a set of three multilayer coils symmetrically mounted on the rotary frame to increase the partition efficiency and sample loading capacity of the system. The above design also provides a perfect balance of the centrifuge system, once the hydrodynamic equilibrium is established in the separation column. In Part I[1], the potential capability of the present apparatus has been demonstrated in separation of 2,4-dinitrophenyl (DNP) amino acids with a solvent system composed of chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1, v/v/v).

This paper describes the applications of this new apparatus in semipreparative separation of various biological samples with two-phase solvent systems covering a broad range of polarity.

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EXPERIMENTAL

Apparatus

The principle of the design and function of the apparatus has been described in detail in Part I[1]. Fig. 1 shows the apparatus which is equipped with three multilayer coils connected in series with flow tubes to make up a total capacity of 400 ml.

Each multilayer coil was prepared from a single piece of about 70 m long × 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing with a standard wall thickness (0.4 mm) (Zeus, Raritan, NJ, U.S.A.) wound onto a holder hub measuring 7.5 cm in diameter. A total of nine layers of coils were formed between a pair of flanges spaced 5 cm apart, resulting in about 200 helical turns with a total capacity of about 135 ml. The β value ($\beta = r/R$, where r is the distance from the axis of the holder to the coil and R the distance between the holder axis and the central axis of the centrifuge) ranges from 0.5 at the internal terminal to 0.75 at the external terminal. In order to prevent dislocation of the multilayer coil on the holder, innermost layer of the coil was glued onto the hub with silicone rubber adhesive while a sheath of heat-shrinkable vinyl tubing was applied over the entire multilayer coil and the peripheral portion of each flange. Three multilayer coils are serially connected with 0.85 mm I.D. thick wall (0.46 mm) PTFE tubing as described in Part I.

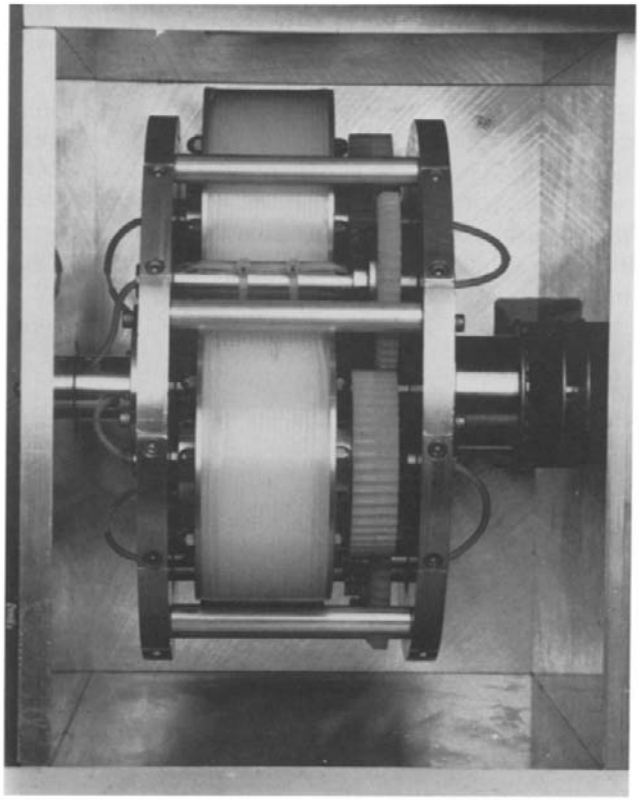

Fig. 1. Photograph of the apparatus.

Reagents

Organic solvents, including *n*-hexane, chloroform, ethyl acetate, *n*-butanol and methanol, were glass-distilled chromatographic grade and obtained from Burdick & Jackson Labs., Muskegon, MI, U.S.A. USP 95% ethanol was obtained from Warner-Graham, Cockeysville, MD, U.S.A. Ammonium acetate was of reagent grade and purchased from Fisher Scientific, Fair Lawn, NJ, U.S.A.

Indole-3-acetamide (IA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-acetonitrile (IAN), bacitracin, oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) were all reagent grade and obtained from Sigma, St. Louis, MO, U.S.A. The crude triterpenoic acids were obtained from the extract of *Boswellia carterii* (Burseraceae). Sea buckthorn (*Hippophae rhamnoides*) ethanol extract dried powder was obtained from China by the courtesy of Professor Tian-You Zhang, Beijing Institute of New Technology Application, Beijing, China.

Preparation of two-phase solvent systems and sample solutions

Six pairs of two-phase solvent systems used in the present study are listed in Table I in the increasing order of polarity. Each solvent mixture was thoroughly equilibrated in a separatory funnel by repeating vigorous shaking and degassing several times at room temperature, and the two phases were separated shortly before use.

For the present study, the following five samples were selected: triterpenoic acids, indole auxins, flavonoids in a crude sea buckthorn ethanol extract, commercial bacitracin, and tetracycline derivatives as listed in Table I. Triterpenoic acids were separated with a two-phase solvent system composed of *n*-hexane–95% ethanol–water (6:5:2, v/v/v), and the sample solution was prepared by dissolving 500 mg of the crude triterpenoic acids in 5 ml of the upper phase of the above solvent system. Indole auxins were separated with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v). The sample solution was prepared by dissolving 100 mg of a sample mixture consisting of 10 mg of IA and 30 mg each of IAA, IBA and IAN in 2 ml of the upper non-aqueous stationary phase. A crude sea buckthorn ethanol extract was separated with a two-phase solvent system composed of chloroform–methanol–water (4:3:2, v/v/v). The sample solution was prepared by first dissolving 100 mg of the dried powder in 1.5 ml of methanol, and then adding 2 ml of chloroform and 1 ml of distilled water to adjust the phase composition. The total sample volume was 4.5 ml consisting of about equal volumes of the upper and the lower phases. The bacitracin was separated with a two-phase solvent system composed of chloroform–95% ethanol–water (5:4:3, v/v/v) and 100 mg of the sample were dissolved in 1 ml of the lower non-aqueous phase. The tetracycline derivatives were separated with two different solvent systems, *i.e.*, ethyl acetate–*n*-butanol–water (2:3:5, v/v/v) and ethyl acetate–*n*-butanol–0.25 *M* ammonium acetate (1:1:2, v/v/v), and for each solvent system sample solution was prepared by dissolving a total 50 mg quantity of the mixture consisting of 10 mg of OTC, 20 mg of CTC and 20 mg of DC in 5 ml of the phase mixture.

Separation procedure

The HSCCC separations were performed according to the standard preparative CCC method, as described elsewhere[1,2], in the following manner. In each separation,

TABLE I

SUMMARY OF EXPERIMENTAL CONDITIONS

Sample	*Amount (mg)*	*Solvent system*		*Mobile phase*	*Flow-rate (ml/h)*	*Revolution (rpm)*	*Pressure (p.s.i.)*	*Retention (%)*
Triterpenoic acids	500	*n*-Hexane	6	Lower aqueous	180	1200	90	70.0
		95% Ethanol	5					
		Water	2					
Indole auxins								
Indole-3-acetamide	10	*n*-Hexane	1	Lower aqueous	600	1250	260	57.4
Indole-3-acetic acid	30	Ethyl acetate	1					
Indole-3-butyric acid	30	Methanol	1					
Indole-3-acetonitrile	30	Water	1					
Flavonoids		Chloroform	4	Lower non-aqueous	360	1250	130	51.3
(see buckthorn ethanol extract)	100	Methanol	3					
		Water	2					
Bacitracin	50	Chloroform	5	Lower non-aqueous	360	1250	170	49.1
		95% Ethanol	4					
		Water	3					
Tetracycline derivatives								
Oxytetracycline	10	Ethyl acetate	2	Lower aqueous	300	1250	80	59.2
Chlortetracycline	20	*n*-Butanol	3					
Doxycycline	20	Water	5					
Oxytetracycline	10	Ethyl acetate	1	Lower aqueous	360	1250	90	64.0
Chlortetracycline	20	*n*-Butanol	1					
Doxycycline	20	0.25 *M* Ammonium acetate	2					

the column was first entirely filled with the stationary (upper) phase. This was followed by injection of sample solution through the sample port. Then, the mobile (lower) phase was introduced into the column in a head-to-tail elution mode, while the apparatus was run at the desired speed of 1200 to 1250 rpm. The effluent from the outlet of the separation column was continuously monitored with an LKB Uvicord S at the suitable wavelength and fractionated with an LKB fraction collector. After the separation was completed, the centrifugation was stopped and the column contents were collected into a graduated cylinder to measure the volume of the stationary phase retained in the column. This was done by connecting the inlet of the separation column to a pressured nitrogen line (*ca.* 100 p.s.i.) while the column was rotated at 200–300 rpm in the tail to head elution mode to accelerate the collection process. Then, the column was washed by pumping about 100 ml of ethanol at 10 ml/min while it was rotated at 100–200 rpm in the tail-to-head elution mode. Finally, the column was emptied and dried by passing nitrogen pressured at *ca.* 100 p.s.i.

Analysis of fractions

The fractions collected in each separation were subjected to spectrophotometric analysis to obtain an elution curve. An aliquot of each fraction was mixed with a known volume of methanol or water (used when the effluent is an aqueous phase containing salt) and the absorbance was determined at the suitable wavelengths (210 nm for triterpenoic acids, 250 nm for bacitracin, 260 nm for flavonoids, 280 nm for indole auxins and 350 nm for tetracycline derivatives) with a Zeiss PM6 spectrophotometer.

For indentification, peak fractions of triterpenoic acids and bacitracin components (A and F) were further separated by high-performance liquid chromatography (HPLC) and retention times compared to those of known samples. These HPLC analyses were performed by using a set of Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of a variable-wavelength UV detector (Model SPD-6A), a constant-flow pump (Model LC-6A) with a manual injector kit and a recording data processor (Model C-R5A). A Capcell Pak C_{18} column (15 × 0.46 cm I.D., type AG) (Shiseido, Tokyo, Japan) was employed. The HPLC analysis of triterpenoic acids was performed by isocratic elution of the mobile phase composed of methanol–distilled water (95:5, v/v) at a flow-rate of 1.5 ml/min and the effluent was monitored at 210 nm. Bacitracin fractions were similarly analyzed with a mobile phase composed of methanol and 0.04 *M* Na_2HPO_4 (pH 9.4) at a flow-rate of 1 ml/min and the effluent was monitored at 234 nm.

The peaks for isorhamnetin and quercetin in the flavonoid separation were identified previously by comparing the retention times to those obtained from the pure compounds[3].

RESULTS AND DISCUSSION

Preparative capability of the present HSCCC system was demonstrated in separations of five sets of samples with suitable two-phase solvent systems having a wide range of polarity. Separations were performed under optimized experimental conditions, as summarized in Table I, where experiments were arranged in the increasing order of polarity of the solvent system used for separation.

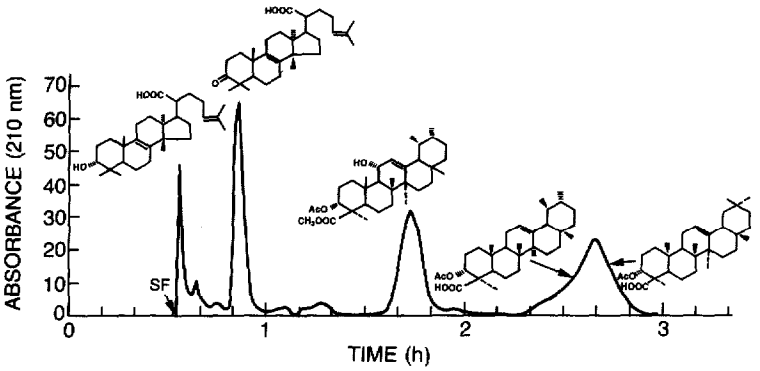

Fig. 2. Chromatogram of triterpenoic acid obtained from the present HSCCC system. See Table I for experimental conditions. Ac = Acetyl; SF = solvent front.

Fig. 2 shows a chromatogram of triterpenoic acids obtained with a two-phase solvent system composed of *n*-hexane–95% ethanol–water (6:5:2, v/v/v) at a flow-rate of 3 ml/min, using a lower aqueous phase as the mobile phase. A total of 500 mg of the crude sample was efficiently separated in multiple peaks within 3 h of elution. Four triterpenoic acids corresponding to peaks 1 to 4 were further separated and retention lines compared to known triterpenoic acids by HPLC analysis.

Fig. 3 shows a chromatogram of indole auxins obtained with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v) using the lower aqueous phase as the mobile phase. Four components with a total weight of 100 mg of the synthetic mixture were well resolved in symmetrical peaks and eluted out within 1 h. The partition efficiency of each peak was estimated according to the following conventional gas chromatographic formula:

$$N = (4R/W)^2 \tag{1}$$

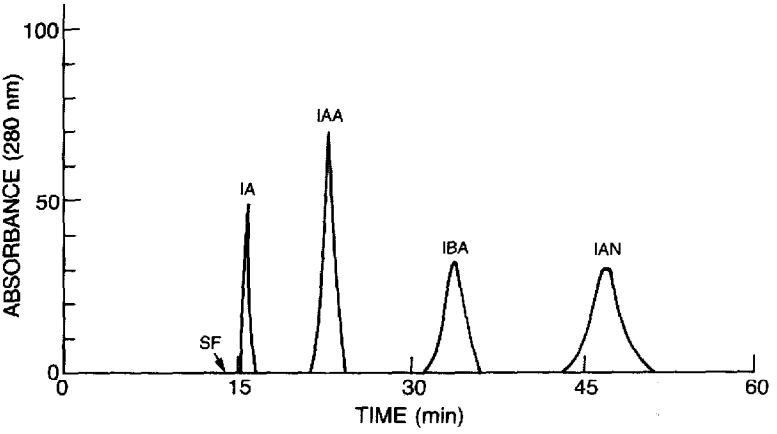

Fig. 3. Chromatogram of indole auxins obtained from the present HSCCC system. Peaks: IA = indole-3-acetamide; IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; IAN = indole-3-acetonitrile. See Table I for experimental conditions.

where N denotes the partition efficiency expressed in terms of theoretical plates (TP); R, the retention time or volume referred to the peak maximum; and W, the peak width expressed in the same unit as R. The results gave high partition efficiencies ranging from 3500 TP for the first peak to 1200 TP for the fourth peak.

Fig. 4 shows the separation of a commercial bacitracin sample with a two-phase solvent system composed of chloroform–95% ethanol–water (5:4:3, v/v/v) by eluting the lower non-aqueous phase at a high flow-rate of 6 ml/min. A total 100 mg quantity of the bacitracin sample was separated in 2 h. Bacitracin A was found in the major peak which showed a high purity as indicated by HPLC analysis of the peak fraction.

Flavonoids in a crude ethanol extract of sea buckthorn were separated with a two-phase solvent system composed of chloroform–methanol–water (4:3:2, v/v/v) with the lower non-aqueous phase used as the mobile phase. Fig. 5A shows a chromatogram of the flavonoids which revealed one major peak of isorhamnetin and several minor peaks including the quercetin peak. The partition efficiency of the major peak was computed as 2150 TP. Fig. 5B illustrates a similar chromatogram obtained from a commercial high-speed CCC centrifuge of 10 cm revolution radius equipped with a single multilayer coil column of 1.6 mm I.D. and a 280 ml capacity[3]. The separation was performed with the same solvent system under the similar operational conditions except at a lower flow-rate of 3 ml/min and at a lower revolutional speed of 800 rpm. The partition efficiency computed from the isorhamnetin peak was 800 TP. Comparison between these two chromatograms (Fig. 5A and B); revealed that the present system yields more efficient separation in a shorter elution time. A near three-fold increase in partition efficiency in the present model is attributable to various factors such as the increased column length (about 1.5 times that in the existing model), reduced helical diameter, and higher revolutional speed (1200 rpm *vs.* 800 rpm).

Fig. 6A and B show chromatograms of three tetracycline derivatives, oxytetracycline (10 mg), chlortetracycline (20 mg) and doxycycline (20 mg), obtained with

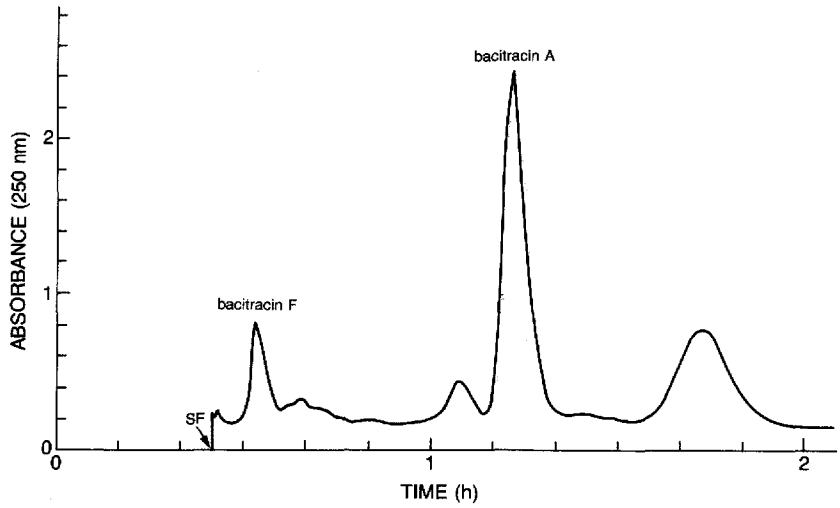

Fig. 4. Chromatogram of bacitracin components obtained with the present HSCCC system. See Table I for experimental conditions.

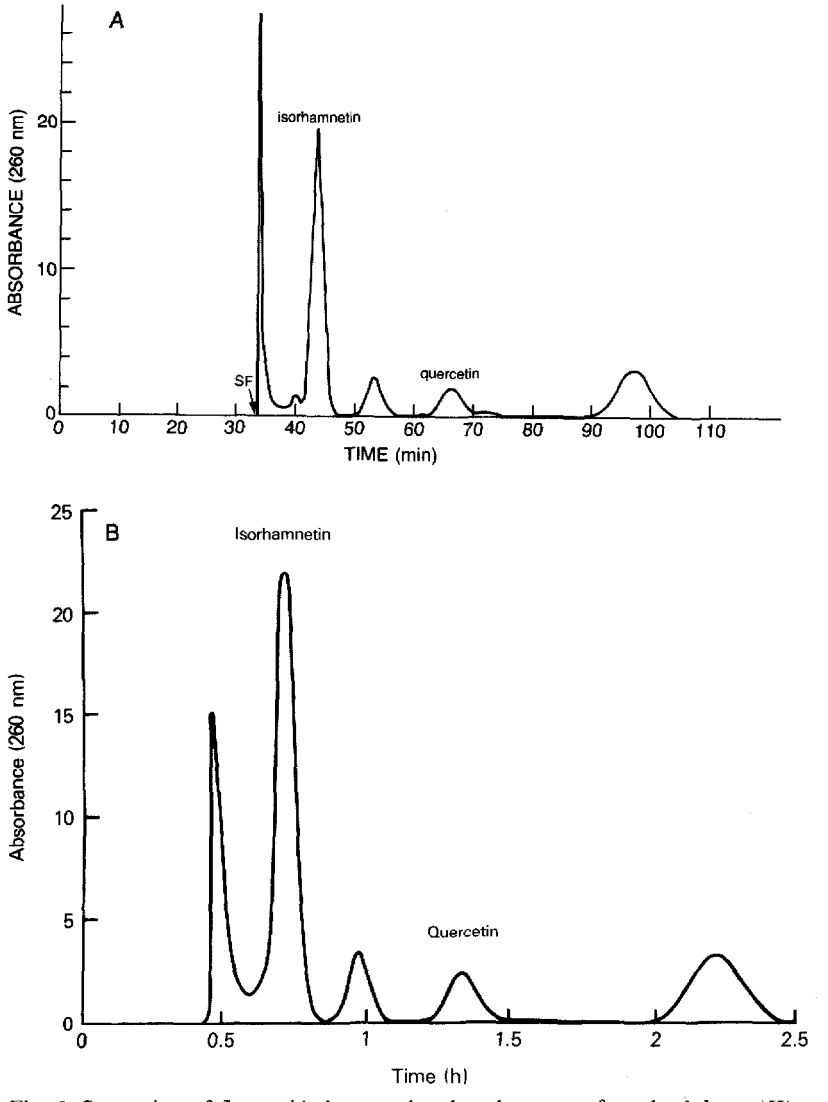

Fig. 5. Separation of flavonoids in a crude ethanol extract of sea buckthorn (*Hippophae rhamnoides*) obtained from two different high-speed CCC instruments. (A) Chromatogram obtained with the present HSCCC system. See Table I for experimental conditions. (B) Chromatogram obtained with a commercial apparatus[3]. Experimental conditions are described in the text.

two different solvent systems, *i.e.*, ethyl acetate–*n*-butanol–water (2:3:5, v/v/v) (chromatogram A) and ethyl acetate–*n*-butanol–0.25 *M* ammonium acetate (1:1:2, v/v/v) (chromatogram B). Both separations were performed under similar operational conditions except for a minor difference in the applied flow-rate which was 5 ml/min for A and 6 ml/min for B. Nevertheless, these two chromatograms revealed a striking difference in peak resolution while the relative retention times of the three peaks remain fairly similar. Chromatogram A shows broad peaks, especially for the second and the

third which were only partially resolved. The partition efficiencies computed for the three peaks revealed an extremely low value in the second peak, *i.e.*, 430 TP for the first peak, 38 TP for the second peak and 144 TP for the third peak. In chromatogram B, the separations were remarkably improved, especially in the second and the third peak which are completely resolved by disclosing a shoulder and a fairly well resolved minor peak between them. Partition efficiencies are much improved for the second and the third peaks, *i.e.*, 340 TP for the first peak, 690 TP for the second peak, and 380 TP for the third peak.

The drastically broad peaks of CTC and DC observed in Fig. 6A cannot be explained on the basis of molecular interactions between two different species[4] because each component, if chromatographed separately, reproduces the broad but fairly symmetrical peak at the original location. The possibility of the molecular

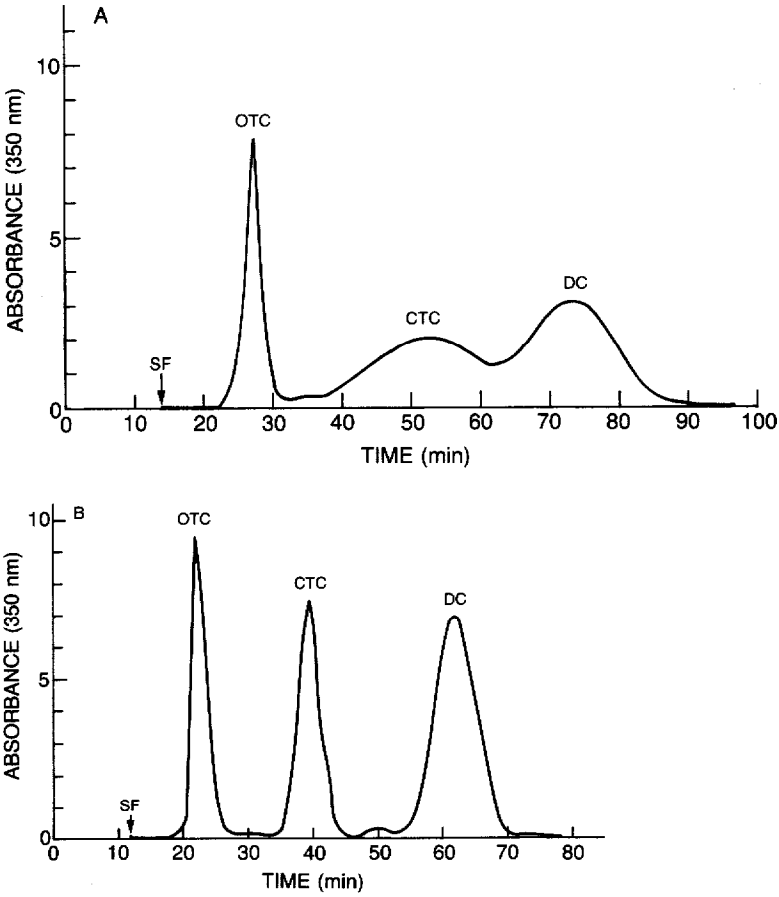

Fig. 6. Separation of tetracycline derivatives obtained by the present HSCCC system with two different solvent systems. (A) Chromatogram obtained with the ethyl acetate–*n*-butanol–water (2:3:5, v/v/v) solvent system. (B) Chromatogram obtained with the ethyl acetate–*n*-butanol–0.25 *M* aqueous ammonium acetate (1:1:2, v/v/v) solvent system. Peaks: OTC = oxytetracycline; CTC = chlortetracycline; DC = doxycycline. See Table I for experimental conditions.

interaction within a single species is also unlikely because molecular aggregation usually causes a skewed peak as often observed in peptide separation.

Although an addition of ammonium acetate to the solvent system remarkably improved the separation, partition efficiencies computed from the obtained chromatogram range from 300 to 700 TP, which are still much lower than those obtained from separations of other samples described earlier. It is conceivable that once the mechanism of the salt effect is fully understood, the partition efficiencies in tetracycline derivative separation may be further improved by selecting an optimum combination of salt and solvent system.

The overall experimental results described above successfully demonstrated excellent capability of the present HSCCC system in semipreparative separations of various biological samples ranging in quantity from 50 to 500 mg. The partition efficiency of the present system may be further increased by the use of smaller I.D. columns. For gram-quantity preparative separations, the column capacity can be increased by mounting larger I.D. columns on wider column holders.

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