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Review

Separation of antibiotics by counter-current chromatography

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Abstract

This paper reviews recent applications of counter-current chromatography (CCC) to the separation of antibiotics. It also covers the recent development of CCC instruments and the optimization of the two-phase solvent system. The CCC technique offers a high resolving power when the proper solvent system is carefully selected and becomes a powerful tool to separate various components from antibiotics complexes. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Counter-current chromatography; Reviews; Solvent systems; Antibiotics

Contents

1. Introduction

Development of antibiotics necessitates isolation and purification of a desired compound from a complicated matrix such as fermentation broth and crude extract. Although recent advances in the HPLC technology using sophisticated equipments and refined adsorbents highly facilitate the isolation of antibiotics, there are some drawbacks due to various complications arising from the use of a solid support.

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Counter-current chromatography (CCC) [1] is a unique form of liquid partition chromatography which utilizes a separation column free of solid support matrix. Because of this support-free system, the method provides an important advantage over other chromatographic methods by eliminating various complications including an adsorptive loss and deactivation of samples, contamination, etc.. During the 1970s, the method was steadily improved by accelerating the separation speed and efficiency. In the early 1980s, an epoch making advance was achieved by the advent of high-speed CCC (HSCCC) which can yield highly efficient separation in a short period of time. Because of its high performance, the recent research and development of the CCC technology have been almost entirely focused on HSCCC.

In this paper, we describe the recent development in CCC instruments and their applications to the separation of antibiotics and demonstrate how to select the two-phase solvent system and optimize its composition.

2. Instruments

The liquid–liquid partition method is an ideal means for separating natural products. In the 1950s the counter-current distribution method (CCD) [2] was widely used for the separation. However, with various disadvantages such as a bulky fragile apparatus, long separation times and excessive dilution of samples, the method was quickly replaced by liquid chromatography. However, CCD provides one important advantage over liquid chromatography in that it uses no solid support, thus eliminating all complications including an adsorptive loss and denaturation of samples and tailing of solute peaks, etc.. In the early 1970s CCC was introduced which utilizes no solid support, as in CCD, and yet enables continuous elution, monitoring and fractionation, as in liquid chromatography. Since then a variety of CCC schemes have been developed and successfully applied for the separation of various natural and synthetic products [3–9]. Below, typical CCC instruments used for purification of antibiotics are described.

2.1. Droplet counter-current chromatography

This simple CCC scheme can perform efficient preparative separations [10] and has been used for purification of various antibiotics such as gramicidins [11], tyrocidines [11] and tetracyclines [12].

The apparatus consists of a set of vertical straight tubes serially connected with narrow transfer tubing. The original CCC apparatus is equipped with 300 glass tubes each 60 cm×1.8 mm I.D.. The total capacity is about 600 ml including the volume in the transfer tubing (about 15% of the total capacity). It can produce 900 theoretical plates. The operation of droplet droplet CCC (DCCC) is initiated by filling the entire column with the stationary phase of an equilibrated two-phase solvent system followed by injection of sample solution. Then, the other phase is introduced into the first unit in such a way that the mobile phase can travel through the column of the stationary phase by the effect of gravity, i.e. the mobile phase is introduced from the bottom if it is the lighter phase, and from the top if it is the heavier phase. Under the optimum flow rate, the mobile phase forms multiple droplets into the stationary phase to divide the column space into numerous partition units and this process is repeated in each partition unit. Consequently, the solutes are separated according to their partition coefficient in a manner analogous to liquid chromatography, but in the absence of solid support.

DCCC necessitates a proper choice of solvent systems for producing a droplet flow of the mobile phase in the column. The most popularly used solvent system is composed of chloroform, methanol and water at various volume ratios. Although a sizable separation usually takes a few days, the system requires no attendance during the separation.

2.2. Horizontal flow-through coil planet centrifuge

This centrifugal CCC system allows the use of a broad spectrum of two-phase solvent systems and produces preparative separations of gram-quantity of compounds [13–15]. The prototype was first fabricated at the machine shop of the Laboratory of Technical Development, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA. The unit provides a synchro-

nous planetary motion of the column holder in such a way that the holder rotates about its own axis during one revolution around the central axis of the centrifuge, both in the same direction. This planetary motion prevents twisting of the flow tubes and therefore eliminates the use of rotary seals which often become a source of leakage and contamination. A set of coiled columns (2.6 mm I.D.) is connected in series with narrow transfer tubes (0.85 mm I.D.) and arranged around the holder. The centrifugal force produced by the planetary motion retains the stationary phase in each helical turn while constantly mixing the two phases to facilitate the partition process. This CCC apparatus was one of the most useful models before the advent of high-speed CCC.

2.3. Multilayer coil high-speed counter-current chromatography apparatus

This highly efficient CCC apparatus was derived from the above horizontal flow-through coil planet centrifuge (CPC) by mounting the coiled column coaxially around the column holder hub [16–21]. This simple alteration of the column orientation radically changes the hydrodynamic motion of the two phases in the coiled column by generating an Archimedean screw force which drives all the objects in the coil competitively toward one end of the coil. This end of the coil is conventionally defined as the head and the other end, the tail. By the effect of the Archimedean screw force, the two immiscible solvent phases present in an end-closed coil undergo rapid and complete separation along the coil in such a way that one phase (head phase) occupies the head side and the other phase (tail phase) the tail side (Fig. 1A). This hydrodynamic motion can be efficiently used for performing CCC by either eluting the head phase through the tail end or the tail phase through the head end of the coil which is previously filled with the stationary phase (Fig. 1B). In both cases the mobile phase can quickly travel through the stationary phase under vigorous mixing while leaving a large volume of the stationary phase in the column. Also the system can be applied to dual CCC by simultaneously introducing the two phases through the respective terminus, i.e. the head phase through the tail and the tail phase through the head. This dual CCC operation necessitates an additional

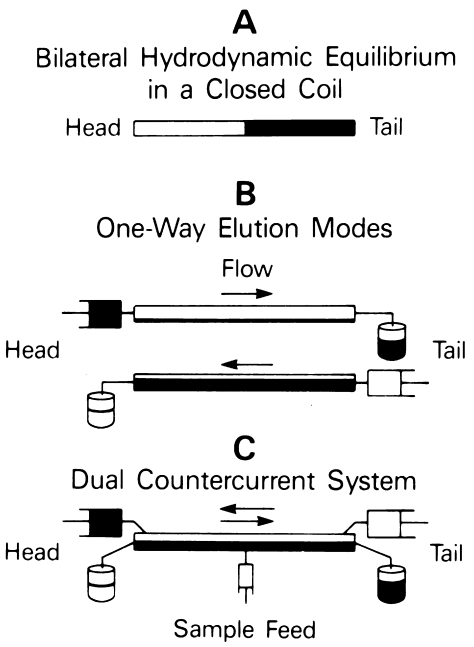


Fig. 1. Mechanism of high-speed CCC. All coils are drawn as straight tubes to indicate the overall distribution of the two immiscible phases. (A) The unilateral hydrodynamic distribution of the two phases in an end-closed coil. (B) Two elution modes for HSCCC. (C) Dual CCC operation which requires five flow tubes.

flow tube in each terminus and if desired the sample feed tube is connected at the middle portion of the coil (Fig. 1C).

The unique hydrodynamic motion of the two immiscible phases in the present system was observed under stroboscopic illumination. Fig. 2 schematically illustrates the hydrodynamic equilibrium of the two solvent phases in the spiral column undergoing the synchronous planetary motion. As shown in the upper diagram, the rotating column is divided into two areas. About one fourth of the area near the center of the centrifuge shows vigorous mixing of the two phases (mixing zone) while in the rest of the area two phases are separated in such a way that the heavier phase occupies the outer portion and the lighter phase in the inner portion of the spiral path. The lower diagram illustrates the motion of the mixing zone through the stretched spiral column corresponding to the positions I through IV in the upper diagram. Each mixing zone travels through the column, one round per one revolution of the column.

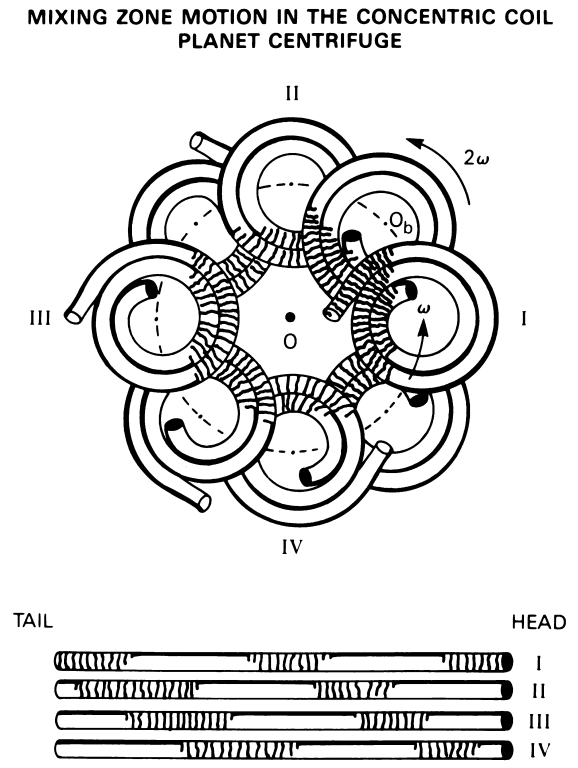


Fig. 2. Hydrodynamic motion of the mixing zone in HSCCC. (Top) Stroboscopic observation of the two phases in the spiral column undergoing a synchronous planetary motion. (Bottom) Motion of the mixing zone through the stretched spiral column.

This indicates that at every portion of the column the two phases are subjected to a partition process of alternating mixing and settling at an enormously high frequency. For example, at 800 rpm the solutes are subjected to a partition cycle at a rate of 13 times per second. Because of the high partition efficiency and speedy separation, this CCC scheme is named high-speed CCC (HSCCC).

In the original prototype of the multilayer coil HSCCC centrifuge [17], the synchronous planetary motion of the column holder is produced by coupling a pair of identical gears, the planetary gear on the holder shaft and the stationary sun gear on the central axis of the centrifuge. The coiled column was prepared by winding a single piece of PTFE tubing (130 m×1.6 mm I.D.) directly onto the holder hub (5 cm diameter) making multiple coiled layers between a pair of flanges spaced 5 cm apart. A counterweight was mounted on the opposite side of the rotary frame to balance the centrifuge system. For an analytical separation, a miniature HSCCC model was constructed [22]. It holds a column holder and a counterweight symmetrically at a distance of 2.5 cm from the central axis of the centrifuge. The multilayer coil separation column consists of 15 m×0.85 mm I.D. PTFE tubing with a total capacity of about 8 ml. The maximum rotation speed is 4000 rpm (440 *g*).

More recently the design of the apparatus was improved by eliminating the counterweight, as shown in Fig. 3. The original model in the top diagram holds a column holder and a counterweight on the rotary frame. In the lower diagram the newly designed CCC unit holds two or more column holders symmetrically around the centrifuge frame to achieve a perfect balance of the centrifuge. All columns are connected in series with a transfer tube to increase the partition efficiency and the sample loading capacity. The improved model of the HSCCC centrifuge is equipped with three column holders [23]. The multilayer coil for analytical separation consists of 100 m×1 mm I.D. PTFE

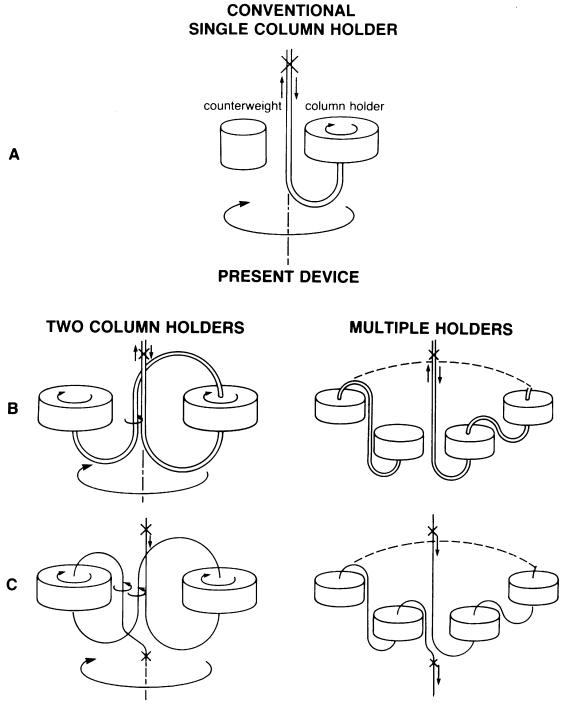


Fig. 3. Improved design of the HSCCC centrifuge.

tubing with a 90 ml capacity. Three columns are connected in series to triple the capacity to 270 ml. The system is capable of producing a high partition efficiency of several thousand theoretical plates in several hours. A large preparative model of the HSCCC centrifuge similarly holds three multilayer coils, each consisting of 100 m×2.6 mm I.D. PTFE tubing with a capacity of 530 ml [24]. Up to 20 g of a crude sample can be loaded and it is ideal for natural product separations.

2.4. Foam counter-current chromatography centrifuge

One variety of the HSCCC centrifuge is designed for dual CCC (see Fig. 1C) [25]. The prototype of the foam CCC apparatus holds a large column holder and a counterweight holder symmetrically at 20 cm from the central axis of the centrifuge. The design of the coiled column is illustrated in Fig. 4. The coiled column is equipped with five flow tubes as indicated. Simultaneous introduction of N_2 and the liquid phase through the respective flow tube produces a counter-current between the gas and liquid phase through the coil. Consequently, the sample mixture injected through the middle portion of the column is separated according to the foaming capability: the foam active components travel through the coil with the gas phase and elute through the foam collection line, whereas the rest of the components moves with the liquid phase and elutes through the liquid collection line. Bacitracins were fractionated using this apparatus as described later.

2.5. Cross-axis coil planet centrifuge

This seal-free flow-through CCC centrifuge is considered as one variety of HSCCC but based on a different type of synchronous planetary motion [26–28]. The apparatus produces a planetary motion in such a way that the coil holder revolves around the central axis of the centrifuge and synchronously rotates about its horizontally oriented axis. Accordingly, the axes of the rotation and revolution form a 90° angle as suggested by its name. In the later design of the prototype, the position of the column holder is shifted along the rotary shaft from its midpoint so that a portion of the centrifugal force vector acts across the diameter of the coiled tube [29–31]. This prevents emulsification of the two phases which may produce adverse effects for the retention of the stationary phase. Thus, the cross-axis (X-axis) CPC ensures stable retention of the stationary phase and it is particularly suited for the large preparative separation of crude samples.

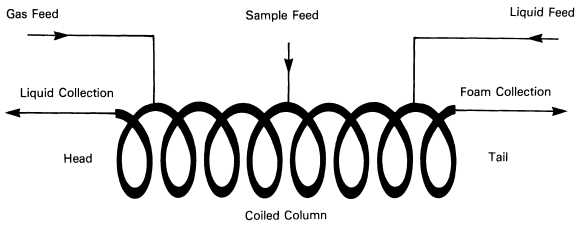


Fig. 4. Design of the coiled column for foam CCC.

3. Selection and optimization of the two-phase solvent system

CCC utilizes two immiscible solvent phases, one as a stationary phase and the other as a mobile phase. Solutes are subjected to a continuous partition process between these two phases in a column space free of solid support, hence the separation is almost entirely governed by the difference between their partition coefficient, i.e. solute distribution ratio between the stationary and the mobile phases. Consequently, the successful separation necessitates the careful search for a suitable two-phase solvent system to provide an ideal range of the partition coefficients for the applied sample.

Generally speaking, the two phase solvent system should satisfy the following requirements:

1. For ensuring the satisfactory retention of the stationary phase, the settling time of the solvent system should be considerably shorter than 30 s. The settling time is measured as follows: A 2 ml volume of each phase from the equilibrated solvent system is delivered into a 5 ml capacity graduated glass cylinder which is then sealed with a stopper. The solvent in the cylinder is gently mixed by inverting it 5 times and the cylinder is immediately placed on a flat table to measure the time required for the solvent mixture to settle into two layers.

2. For efficient separation, the partition coefficient (K) of the target compound(s) should be close to 1, and the separation factor (α) between the components should be larger than 1.5. If $K<<1$, the solutes are eluted close together near the solvent front resulting in a loss of peak resolution and, if $K>>1$, the solutes are eluted in excessively broad peaks and require a long elution time. The minimum α value of 1.5 is required for the baseline separation in semipreparative CCC equipment providing a moderate partition efficiency of around 800 theoretical plates. The K value for a pure compound can be determined simply by measuring the UV absorbance of each phase after partitioning between the two phases. When the compounds to be separated are not available in pure form, as in most cases for antibiotics, the following method should be used: After partitioning the sample mixture between the two solvent phases, aliquots of the upper and the lower phases are analyzed by HPLC. From these two chromatograms, the K value of each component is determined by comparing the peak heights (or areas) between the corresponding peaks.
3. In addition to the above two major requirements, it is desirable that the solvent system provides nearly equal volumes of each phase to avoid excessive waste of the solvent.
4. Also, it is convenient to use a volatile solvent system: The pure compound is obtained simply by evaporating the collected fractions.

By keeping the above in mind, the following two series of solvent systems can provide an ideal range of the K values for a variety of samples (Tables 1 and 2) [6,32]: *n*-hexane–ethyl acetate–*n*-butanol–methanol–water and chloroform–methanol–water. In each solvent series, the partition coefficient of the

Table 2
Chloroform–methanol–water system

Chloroform–methanol–water	Volume ratio (U/L)[a]	Settling time (s)
10:0:10	0.98	8
10:1:9	1.00	8
10:2:8	1.03	12
10:3:7	1.06	13
10:4:6	1.10	12
10:5:5	1.16	10
10:6:4	1.37	11
10:7:3	2.05	22
10:8:2	–[b]	–[b]

[a] Volume of the upper phase divided by that of the lower phase.
[b] The solvent mixture formed a single phase.

Table 1
n–Hexane–ethyl acetate–butanol–methanol–water system

n-Hexane–ethyl acetate–*n*-butanol–methanol–water	Volume ratio (U/L)[a]	Settling time (s)
10:0:0:5:5	1.05	5
9:1:0:5:5	0.96	8
8:2:0:5:5	0.88	14
7:3:0:5:5	0.82	20
6:4:0:5:5	0.77	22
5:5:0:5:5	0.74	26
4:5:0:4:5	0.80	28
3:5:0:3:5	0.86	30
2:5:0:2:5	0.93	30
1:5:0:1:5	0.92	30
0:5:0:0:5	0.88	32
0:4:1:0:5	0.91	20
0:3:2:0:5	0.99	15
0:2:3:0:5	1.09	12
0:1:4:0:5	1.16	14
0:0:5:0:5	1.22	17

[a] Volume of the upper phase divided by that of the lower phase.

sample can be finely adjusted by modifying the volume ratio of the components.

The first series continuously covers a broad range from a hydrophobic *n*-hexane–water system to a polar *n*-butanol–water system. If the sample requires a more hydrophobic solvent system than hexane–methanol–water (10:5:5, v/v/v), one may reduce the amount of water from the system and/or replace the methanol with ethanol. On the other hand, if a more polar solvent system is needed, the *n*-butanol–water system may be modified by adding a volatile acid or salt, e.g., *n*-butanol–trifluoroacetic acid–water (1:0.001:1 to 1:0.01:1, v/v/v), *n*-butanol–acetic acid–water (4:1:5, v/v/v), and *n*-butanol–0.25 *M* ammonium acetate (1:1, v/v).

In the second series, chloroform–methanol–water provides moderate hydrophobicity. If a satisfactory partition coefficient value cannot be obtained by adjusting the volume ratio of the above system, adding ethanol or replacing methanol with ethanol will increase the hydrophobicity, whereas the partial replacement of chloroform by dichloromethane will increase the polarity of the solvent system.

Most of these two-phase solvent systems provide near 1:1 volume ratios between the upper and the lower phases together with a reasonable range of the settling times in 25 s or less, so that they can be effectively applied to CCC.

4. Counter-current chromatographic separation of antibiotics

CCC has been successfully applied to the separation of various antibiotics (Table 3) [11,12,15,17,33–51], including various peptide antibiotics which are strongly adsorbed to silanol groups on silica gel used in the stationary phase in column chromatography. Applicable sample size is dependent upon the capacity of the CCC column varying from 1 mg to 5 g. The two-phase solvent systems should be selected according to the hydrophobicity of the antibiotics, i.e. *n*-butanol solvent systems for polar groups, chloroform solvent systems for moderate hydrophobic groups and *n*-hexane solvent systems for most hydrophobic groups. In this section, we describe the CCC separation of selected antibiotics such as sporaviridins, bacitracins, colistins and ivermectins, especially focusing on the procedures for optimization of two-phase solvent systems.

The apparatus used in the following studies was a HSCCC-1A prototype multilayer coil planet centrifuge (Shimadzu, Kyoto, Japan) with a 10 cm orbital radius which produces a synchronous planetary motion at 800 rpm. The multilayer coil was prepared by winding a ca. 160 m length of PTFE tubing onto the column holder. Other important factors affecting the separation are as follows: revolution: 800 rpm; stationary phase: upper organic phase; flow rate: 3 ml/min; elution mode: head to tail.

4.1. Sporaviridins

Sporaviridins (SVD, Fig. 5) [41] are basic water soluble antibiotics produced by *Kutzneria viridogrisea* (the previous name; *Streptosporangium viridogriseum*) and they are active against Gram-positive bacteria, acid-fast bacteria and trichophyton [52]. As shown in Fig. 6, they consist of six components and each has a 34-membered lactone and seven monosaccharide units, a pentassacharide (viridopentaose) and two monosaccharides.

The SDV complex is only soluble in polar solvents such as water, methanol and *n*-butanol, and they are extracted with *n*-butanol from the fermentation broth. Therefore, a two-phase solvent system containing *n*-butanol was mainly examined. The SVD complex was entirely partitioned into the upper organic phase, where a mixture of *n*-butanol–water was used as a two-phase solvent system (Table 4). This result indicates that the solubility of the *n*-butanol phase must be decreased to obtain the partition coefficient of 1 and a nonpolar solvent such as *n*-hexane and diethyl ether was added to the *n*-butanol–water solvent system as a modifier. Initially, volumes of *n*-butanol and water were fixed to 10 ml and that of diethyl ether was varied, so that a two-phase solvent system of *n*-butanol–diethyl ether–water (10:4:10, v/v/v) was selected. Next, the volumes of *n*-butanol and diethyl ether were fixed and that of water was varied from 11 to 14. At the solvent ratio of 10:4:12, the almost equally dispersed partition coefficients among six components were obtained (Table 4). This

Table 3
Separation of antibiotics by CCC

Sample	Amount (mg)	Instrument	Solvent system	Mobile phase	Published year	Ref.
Gramicidins A, B and C (peptide antibiotics)	53.3	DCCC	Benzene–chloroform–methanol–water (15:15:23:7, v/v/v/v)	LP	1974	[11]
Tyrocidines (peptide antibiotics	50	DCCC	Chloroform–methanol–0.1 *M* HCl (19:19:12, v/v/v)	LP	1974	[11]
Echinomycin/quinomycin (quinoxaline antibiotics)	1	CPC	Acetone–water–heptane–ethyl acetate (3:1:1:1, v/v/v/v)	LP	1978	[33]
Triostin A/echinomycin (quinoxaline antibiotics)	4	CPC	Trichloroethane–methanol–water (7:3:1, v/v/v)	UP	1978	[33]
Daunorubicin derivatives		HSCCC	Chloroform–ethylene chloride–hexane–methanol–water (1:1:1:3.5:1, v/v/v/v/v)	UP	1981	[34]
Gramicidins A, B and C	100	HSCCC	Benzene–chloroform–methanol–water (15:15:23:7, v/v/v/v)	UP	1982	[17]
Oxytetracycline/chlortetracycline	5	CPC	*n*-Butanol–0.01 *M* HCl (1:1, v/v)	LP	1984	[15]
Tetracycline/impurities	2.5	CPC	Nitromethane–chloroform–pyridine–0.1 *M* EDTA (pH 7) (20:10:3:33, v/v/v/v)	LP	1984	[15]
Trichomycin (polyene antibiotics)	3	CPC	Chloroform–methanol–borate buffer (4:4:3, v/v/v)	UP	1984	[15]
Globoroseamycin (polyene antibiotics)	5–400	CPC	Chloroform–methanol–borate buffer (4:4:3, v/v/v)	UP	1984	[15]
Nystatin	6	CPC	Chloroform–methanol–borate buffer (2:4:3, v/v/v)	LP	1984	[15]
Erythromycin	6	CPC	Methyl isobutyl ketone–acetone–0.2 *M* phosphate/citrate buffer (pH 6.5) (20:1:21, v/v/v)	LP	1984	[15]
Kangdisu/colistin E (peptide antibiotics)	6	CPC	*n*-Butanol–2% dichloroacetic acid (5% sodium chloride) (6:7, v/v)	LP	1984	[15]
Doxorubicin /daunorubicin/their metabolites		CPC	*n*-Butanol–0.3 *M* disodium hydrogen phosphate		1984	[35]
Tetracyclines		DCCC	Chloroform–methanol–*n*-propanol–0.01 *M* HCl (9:12:1:8, v/v/v/v)		1984	[12]
Siderochelin A	400	HSCCC	Chloroform–methanol–water (7:13:8, v/v/v)	UP	1985	[36]
Efrotomycin	670	HSCCC	Carbon tetrachloride–chloroform–methanol–water (5:5:6:4, v/v/v/v)	UP	1985	[36]
Pentalenolactone	50	HSCCC	Chloroform–methanol–water (1:1:1, v/v/v)	UP	1985	[36]
Bu 2313B	200	HSCCC	*n*-Hexane–dichloromethane–methanol–water (5:1:1:1, v/v/v/v)	LP	1985	[36]

Table 3 (Continued)

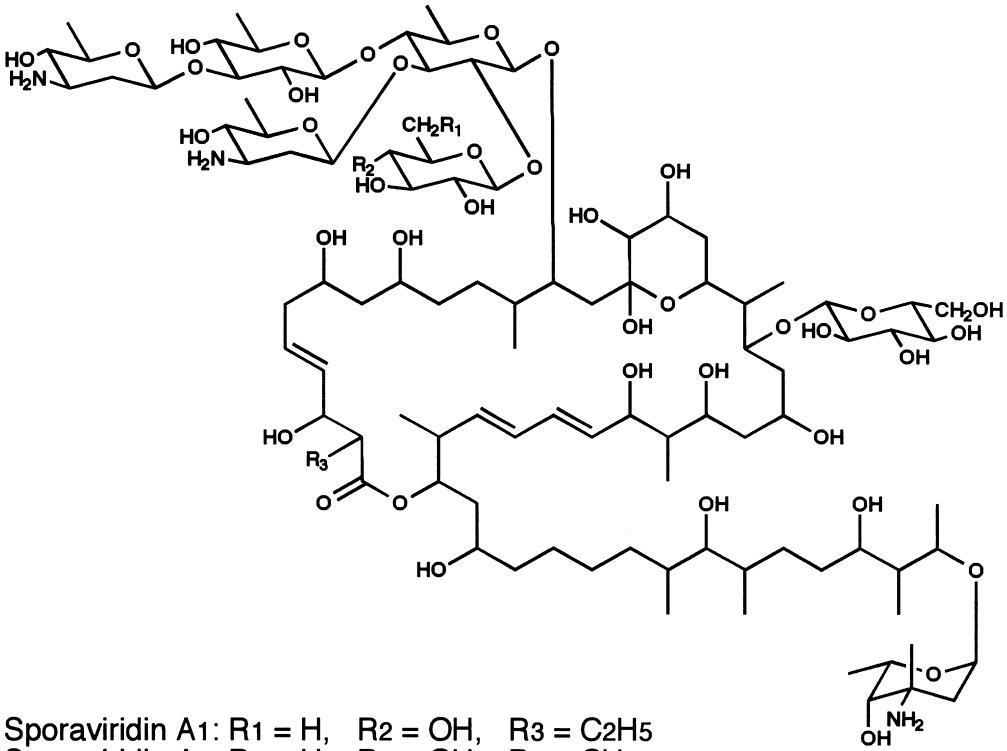
Sample	Amount (mg)	Instrument	Solvent system	Mobile phase	Published year	Ref.
A 201E	350	HSCCC	Carbon tetrachloride–chloroform–methanol–water (2:5:5:5, v/v/v/v)	UP	1985	[36]
Tirandamycin A and B	134	HSCCC	*n*-Hexane–ethyl acetate–methanol–water (70:30:15:6, v/v/v/v)	UP	1985	[36]
Actinomycin complex	83	HSCCC	Diethyl ether–hexane–methanol–water (5:1:4:5, v/v/v/v)	UP	1986	[37]
Candicidin (polyene macrolide antibiotics	100	HSCCC	Chloroform–methanol–water (4:4:3, v/v/v)	UP	1987	[38]
2-Norerythromycins (macrolide antibiotics)	500	HSCCC	*n*-Heptane–benzene–acetone–isopropanol–0.01 *M* citrate buffer (pH 6.3) (5:10:2:3:5, v/v/v/v/v)	UP	1988	[39]
Niddamycins (macrolide antibiotics)	200	HSCCC	Carbon tetrachloride–methanol–0.01 *M* potassium phosphate bussfer (pH 7) (2:3:2, v/v/v)	UP	1988	[39]
Tiacumicins (macrolide antibiotics)	200	HSCCC	Carbon tetrachloride–chloroform–methanol–water (7:3:7:3, v/v/v/v)	UP	1988	[39]
Coloradocin (macrolide antibiotics)	400	HSCCC	Chloroform–methanol–water (1:1:1, v/v/v)	UP	1988	[39]
Bacitracin complex	5	Foam CCC	N_2 gas, water	–	1989	[40]
Bacitracin complex	5000	X-axis CCC	Chloroform–95% ethanol–water (5:4:3, v/v/v)	LP	1989	[30]
Sporaviridin complex	100	HSCCC	*n*-Butanol–diethyl ether–water (10:4:12, v/v/v)	LP	1990	[41]
Bacitracin complex	125 mg/2.5 l	Foam CCC	N_2 gas, water	–	1991	[42]
Bacitracin complex	50	HSCCC	Chloroform–ethanol–water (5:4:3, v/v/v)	LP	1991	[43]
Bacitracin complex	50	HSCCC	Chloroform–ethanol–methanol–water (5:3:3:4, v/v/v/v)	LP	1991	[43]
Mycinamicins		Analytical HSCCC	*n*-Hexane–ethyl acetate–methanol–8% aq. ammonia (1:1:1:1, v/v/v/v)	LP	1991	[44]
Colistin		Analytical HSCCC	*n*-Butanol–0.04 *M* TFA (1:1, v/v) containing 1% glycerol	LP	1991	[44]
Pristinamycns (macrolide antibiotics)	1	HSCCC	Chloroform–ethyl acetate–methanol–water (3:1:3:2, v/v/v/v)	UP	1992	[45]
Pristinamycins (macrolide antibiotics)	1	HSCCC	Chloroform–ethyl acetate–methanol–water (2.4:1.6:3:2, v/v/v/v)	UP	1992	[45]
Ivermectin	25	HSCCC	*n*-Hexane–ethyl acetate–methanol–water (19:1:10:10, v/v/v/v)	LP	1996	[46]
Colistin	20	HSCCC	*n*-Butanol–0.04 *M* TFA (1:1, v/v)	LP	In press	[47]

(Continued overleaf)

Table 3 (Continued)

Sample	Amount (mg)	Instrument	Solvent system	Mobile phase	Published year	Ref.
Benzanthrins A and B (quinone antibiotics)	620	HSCCC	Carbon tetrachloride–chloroform–methanol–water (4:1:4:1, v/v/v/v)	UP	1986	[48]
Niphimycin	700	DCCC	Chloroform–methanol–water (35:65:40, v/v/v)	LP	1983	[49]
Coloradocin	400	HSCCC	Chloroform–methanol–water (1:1:1, v/v/v)	UP	1987	[50]
Dunaimycin (macrolide antibiotics)		HSCCC	*n*-Hexane–ethyl acetate–methanol–water (8:2:10:5, v/v/v/v)/ (70:30:15:6, v/v/v/v)	UP	1991	[51]

DCCC: Doroplet counter-current chromatoraphy.
CPC: Coil planet centrifuge (single layer coiled column).
HSCCC: High-speed counter-current chromatography.



Sporaviridin A1: R1 = H, R2 = OH, R3 = C2H5
Sporaviridin A2: R1 = H, R2 = OH, R3 = CH3
Sporaviridin B1: R1 = H, R2 = NH2, R3 = C2H5
Sporaviridin B2: R1 = H, R2 = NH2, R3 = CH3
Sporaviridin C1: R1 = OH, R2 = OH, R3 = C2H5
Sporaviridin C2: R1 = OH, R2 = OH, R3 = CH3

Fig. 5. Structures of sporaviridins.

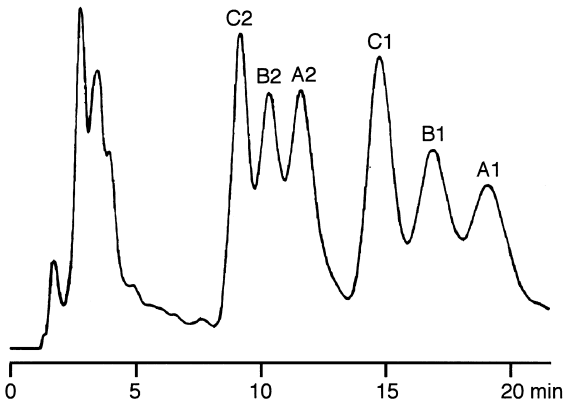


Fig. 6. HPLC separation of sporaviridins. Column: Cosmosil $5C_{18}$ (5 μm, 150 mm×4.6 mm), mobile phase: methanol–1 *M* ammonium chloride (74:26, v/v), flow rate: 1 ml/min, detection: 232 nm.

solvent system was selected for the separation of the components of SVD.

The preparative separation of six components from the SVD complex by HSCCC was performed. In this experiment, the retention of the stationary phase, elution time and elution volume were 75%, 3.5 h and 500 ml, respectively. The six components were eluted in the order of their partition coefficients yielding pure components A1 (1.4 mg), A2 (0.6 mg), B1 (0.7 mg), B2 (0.5 mg), C1 (1.1 mg) and C2 (1.4 mg) from 15 mg of the SVD complex. HPLC analysis of the purified components are shown in Fig. 7.

4.2. Bacitracins

Bacitracins (BCs) [43] are peptide antibiotics produced by *Bacillus subtilis* and *Bacillus licheniformis*. They exhibit an inhibitory activity against Gram-positive bacteria and among the most commonly used antibiotics as animal feed additives [53]. Over twenty components are contained in the bacitracin complex (Fig. 8) among which the major antimicrobial components are BCs-A and -B. BC-F is a degradation product and has nephrotoxicity. Only the structure of BCs-A and F have been determined (Fig. 9).

We selected *n*-butanol, ethyl acetate and chloroform as the organic components and then prepared two-phase solvent systems composed of one of these three solvents and water and/or methanol, which dissolved the bacitracin complex. For the *n*-butanol system, peaks 13–18 in Fig. 8 have suitable partition coefficients, however, those of peaks 20–22 are too high. The ethyl acetate, ethanol and water system showed too long a settling time suggesting a poor retention of the stationary phase. Addition of inorganic salts, such as sodium chloride, potassium chloride or ammonium chloride did not improve the *K* values.

Chloroform, ethanol and/or methanol, water systems are summarized in Table 5. Among all combinations, chloroform–ethanol–methanol–water (5:3:3:4, v/v/v/v) and chloroform–ethanol–water (5:4:3, v/v/v) gave the best *K* values.

Fig. 10 shows the counter-current chromatogram

Table 4
Partition coefficients of SVD components with *n*-butanol systems

n-Butanol–diethyl ether–water	Partition coefficients (U/L)					
	C2	B2	A2	C1	B1	A1
10:0:10	2.96	6.41	6.65	4.87	8.81	9.09
10:3:10	0.96	2.17	2.78	1.84	3.34	4.19
10:4:10	0.50	1.12	1.59	1.04	1.85	2.91
10:5:10	0.38	0.78	1.11	0.74	1.25	2.12
10:6:10	0.39	0.90	1.19	0.81	1.39	1.70
10:7:10	0.24	0.63	1.10	0.59	1.08	1.82
10:4:11	0.31	0.89	1.24	0.70	1.50	2.00
10:4:12	0.38	1.09	1.41	0.80	1.85	2.32
10:4:13	0.37	1.05	1.51	0.73	1.58	2.10
10:4:14	0.29	1.09	1.17	0.57	1.22	1.74

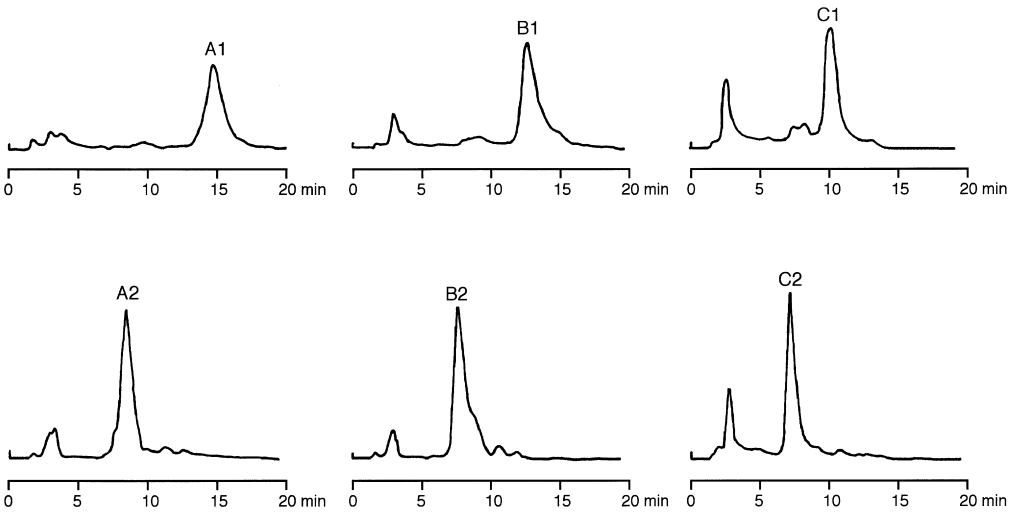


Fig. 7. HPLC separation of each sporaviridin component. Column: Cosmosil $5C_{18}$ (5 μm, 150 mm×4.6 mm), mobile phase: methanol–1 *M* ammonium chloride (76:24, v/v), flow rate: 1 ml/min, detection: 232 nm.

of bacitracin components using chloroform–ethanol–methanol–water (5:3:3:4, v/v/v/v). A 50 mg amount of the bacitracin complex was introduced into the coiled column. The retention of the stationary phase was 72.7% and the elution time was about 3 h. The components were eluted in the order of their partition coefficients, isolating peak 18 (5.9 mg, BC-A) and peak 22 (1.5 mg, BC-F). A large amount (5 g) of bacitracin complex was also separated by the cross-axis CPC using the same solvent system in which pure fractions of 1.85 g of BC-A and 0.28 g of BC-F were obtained.

4.3. Ivermectins

Ivermectins B1 [46] are broad spectrum an-

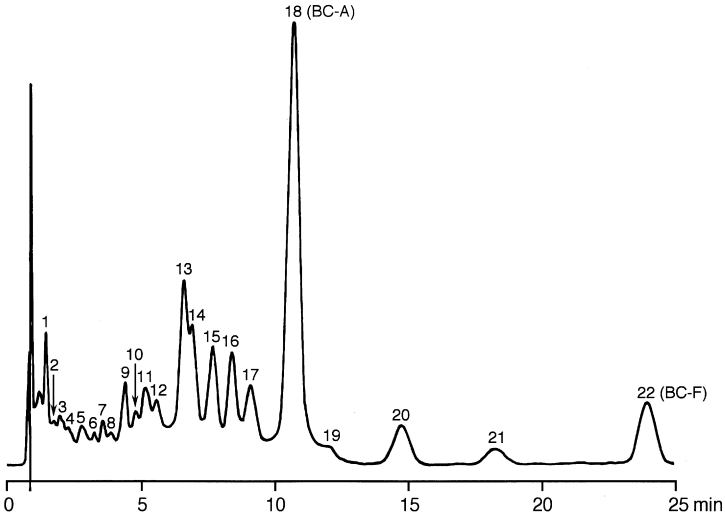


Fig. 8. HPLC separation of bacitracin components. Column: Capcell Pak C_{18} (5 μm, 150 mm×4.6 mm), mobile phase: methanol–0.04 *M* disodium hydrogenphosphate (6:4, v/v), flow rate: 1.3 ml/min, detection: 234 nm.

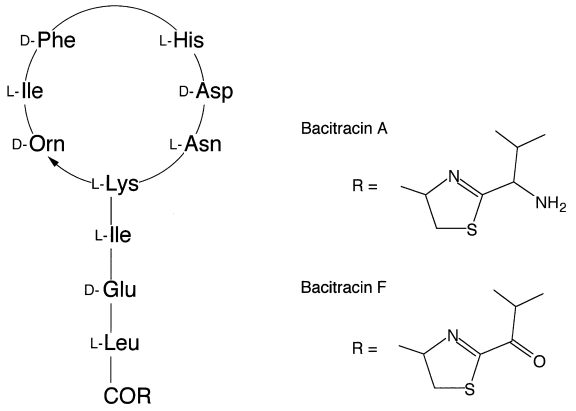


Fig. 9. Structures of bacitracins A and F.

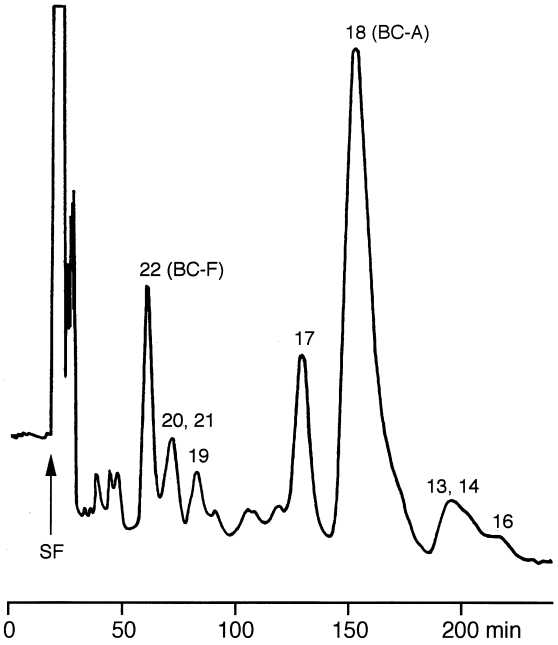


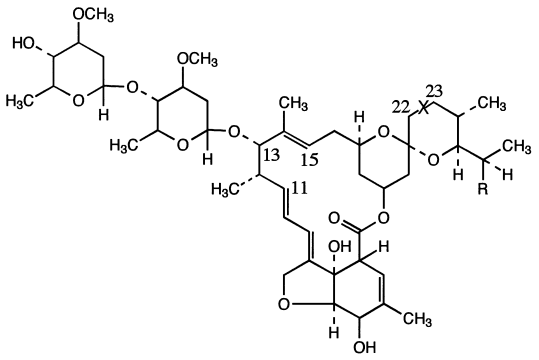
Fig. 10. Counter-current chromatogram of bacitracin components. Solvent system: chloroform–ethanol–methanol–water (5:3:3:4, v/v/v/v), mobile phase: lower phase, flow rate: 3 ml/min, detection: 254 nm.

tiparasitic agents widely used for food-production animals such as cattle, swine and horse. They are derived from the avermectins B1, the natural fermentation products of *Streptomyces avermitilis*. The avermectins B1 have double bonds between carbon atoms at 22 and 23, whereas the ivermectins B1 have single bonds in these positions (Fig. 11) [54]. The ivermectins B1 are a mixture of two major homologs, ivermectin B1a (>80%) and ivermectin B1b (<20%), but a crude ivermectin complex also contains various minor components (Fig. 12A).

We have selected a two-phase solvent system composed of *n*-hexane, ethyl acetate, methanol and water, because it can be used for the separation of components with a broad range of hydrophobicity by modifying the volume ratio of the four solvents. In the *n*-hexane–ethyl acetate–methanol–water (8:2:5:5, v/v/v/v) system first examined, the *K* values of the components corresponding to peaks 1, 2, 3, 4, 5, 6, and 7 were 0, 0.46, 0.61, ∞, 1.86, 3.06 and 4.38, respectively. This indicated that the component of peak 6 (ivermectin B1a) is mostly partitioned in the upper aqueous phase (Table 6). Although the *n*-hexane–ethyl acetate–methanol–water (9:1:5:5, v/v/v/v) system gave an improved *K* value for peak 6, its *K* value was still too large and the difference between peaks 6 and 7 was smaller than 1.5. Finally, a slightly less polar solvent mixture of *n*-hexane–ethyl acetate–methanol–water (19:1:10:10, v/v/v/v) yielded the best *K* values as

Table 5
Partition coefficients of the bacitracin components

n-Butanol–ethanol–methanol–water	Partition coefficients (*U*/*L*)					
	Peaks 3, 14	Peak 17	Peak 18	Peak 20	Peak 21	Peak 22
5:2:3:4	7.20	2.46	4.17	0.64	0.65	0.48
5:2:1:4	∞	∞	33.27	1.62	1.38	0.75
5:3:3:4	3.35	1.40	2.37	0.57	0.47	0.45
5:3:0:3	11.1	3.20	5.34	0.32	0.35	0.27
5:4:0:2	3.19	1.05	2.00	0.25	0.26	0.21
5:4:0:3	5.49	1.46	2.20	0.16	0	0.16
5:4:0:4	6.10	2.04	2.68	0.14	0	0.10



	R	-C_{22}-X-C_{23}-
Ivermectin B1a	C_2H_5	-CH_2-CH_2-
Ivermectin B1b	CH_3	-CH_2-CH_2-
Avermectin B1a	C_2H_5	-CH=CH-
Avermectin B1b	CH_3	-CH=CH-

Fig. 11. Structures of ivermectins and avermectins.

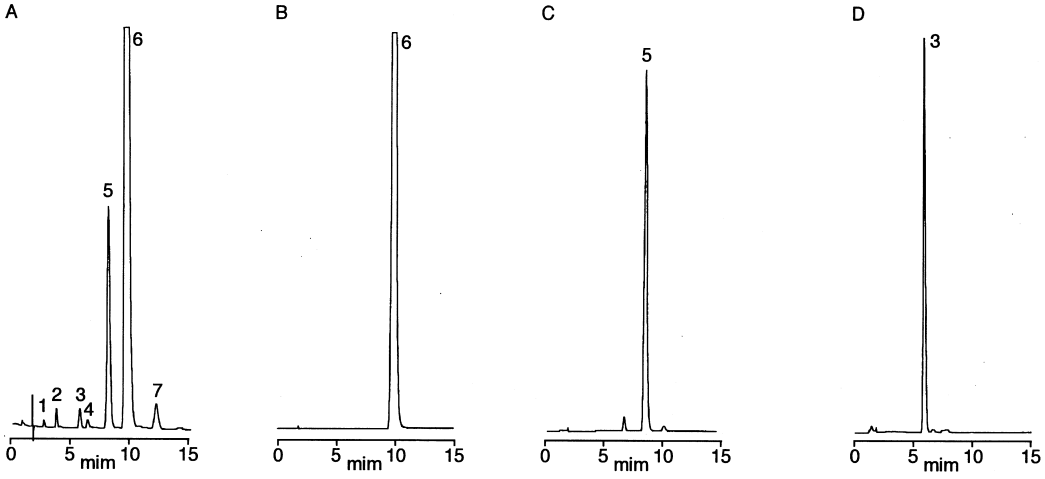


Fig. 12. HPLC separation of ivermectin components. Column: TSK GEL-80 Ts C_{18} (5 μm, 150 mm×4.6 mm), mobile phase: methanol–water (9:1, v/v), flow rate: 1 ml/min, detection: 245 nm. (A) Crude ivermectin. (B) Fraction II (ivermectin B1a). (C) Fraction IV (ivermectin B1a). (D) Fraction VI (avermectin B1a).

indicated in Table 6. The settling time of this solvent system was 7 s and the volume ratio of the upper and the lower phases 0.98. Therefore, this solvent system was selected for the separation of ivermectin components.

A 25 mg quantity of crude ivermectin was sepa-

Table 6
Partition coefficients of the ivermectin components

Solvent system	Peak No.						
	1	2	3	4	5	6	7
n-Hexane–ethyl acetate–methanol–water (8:2:5:5)	0	0.46	0.61	∞	1.86	3.06	4.38
n-Hexane–ethyl acetate–methanol–water (9:1:5:5)	0	0.15	0.33	∞	1.17	2.31	3.21
n-Hexane–ethyl acetate–methanol–water (19:1:10:10)	0	0	0.18	0.48	0.79	1.36	2.83

K=Peak area of upper phase divided by peak area of lower phase.

rated using the above solvent system at a flow rate of 2 ml/min. The retention of the stationary phase was 67.6% and the total separation time was 4.0 h.

The HSCCC elution curve of the ivermectin components monitored at 245 nm is shown in Fig. 13 where the components are separated into three peaks, A, B and C. HPLC analysis of each peak fraction and the column contents revealed that both chromatographic systems elute all components in the same order: HPLC peaks 3, 5 and 6 correspond to HSCCC peaks A, B and C, respectively, and HPLC peak 7 was still retained in the CCC column. This separation yielded 18.7 mg of 99.0% pure ivermectin B1a (Fig. 12B), 1.0 mg of 96.0% pure ivermectin B1b (Fig. 12C) and 0.3 mg of 98.0% pure avermectin B1 a (precursor of ivermectin) (Fig. 12D).

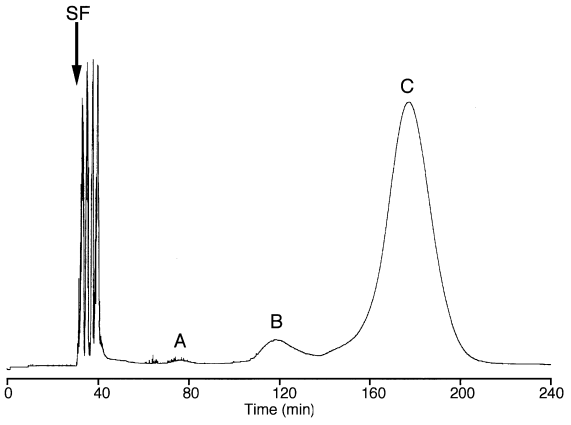


Fig. 13. Counter-current chromatogram of ivermectin components. Solvent system: *n*-hexane–ethyl acetate–methanol–water (19:1:10:10, v/v/v/v), mobile phase: lower phase, flow rate: 2 ml/min, detection: 245 nm.

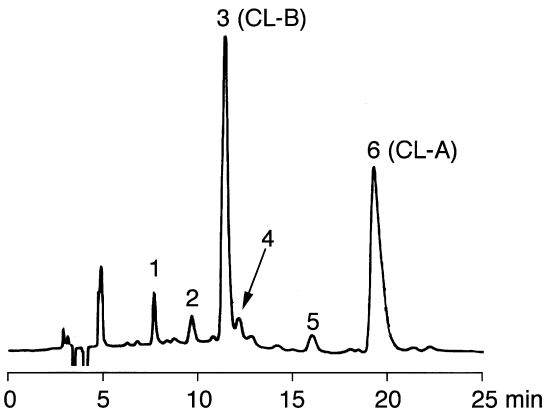


Fig. 14. HPLC profile of commercial CL under the optimal conditions. Column: Chromatorex Ph (5 μm, 250 mm×4.6 mm I.D.), mobile phase: acetonitrile–0.01*M* TFA aq. soln. (24:76, v/v), flow rate: 1.0 ml/min, detection: 210 nm.

4.4. Colistin

Colistin (CL) [44] is a peptide antibiotic produced by *Bacillus polimyxa* var. *Colistinus* that exhibits activity against the Gram-negative organism [55]. CL is a mixture of many components (Fig. 14) and two main components are colistins A (CL-A) and B (CL-B). As shown in Fig. 15, CLs-A and -B are linear-ring peptides and they are different in their N-terminal fatty acid only. CL is used as a feed additive for domestic animals such as calves and swine for preventing bacterial infection and/or improving feed conversion efficiency.

CL is soluble in water, slightly soluble in alcohols but insoluble in nonpolar solvents such as hexane and chloroform. From this property, we selected *n*-butanol and water as a basic solvent system. However, this combination was not suitable by itself, because the CL components were entirely partitioned into an aqueous phase. In order to partition the CL components partly into a butanol phase, various salts (NaCl and Na_2SO_4) or acids [HCI, H_2SO_4 and trifluoroacetic acid (TFA)] were added as the modifier, and the desirable effect was observed from the use of TFA. In addition, the partition of CL com-

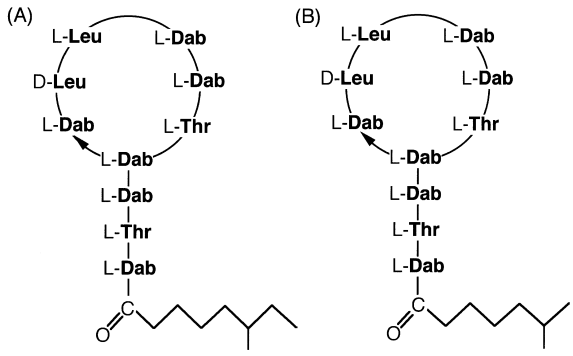


Fig. 15. Structures of colistin components. (A) Colistin A. (B) Colistin B. Dab: α,γ-diaminobutyric acid.

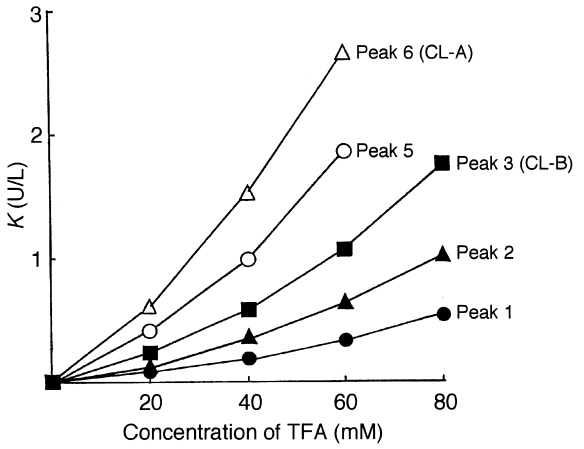


Fig. 16. Influence of TFA concentration on the partition coefficients of CL components.

ponents into the butanol phase increased with the concentration of TFA in the solvent system. This mechanism may be as follows: As shown in Fig. 14, CLs-A and -B have five free amino groups from L-diaminobutyric acid (L-Dab) in their structures and these amino groups dissociate in the aqueous phase. TFA forms an ion pair with those amino groups, so that the hydrophobicity of CL components increases with the concentration of TFA. In order to determine the optimal concentration of TFA in the solvent system. *K* values of 5 components were measured at various TFA concentrations. As shown in Fig. 16, the *K* value of each component increased with the TFA concentration. At 40 m*M* TFA concentration, the *K* values of CL-A and CL-B were 1.5 and 0.6, respectively, while all other *K* values were greater than 1.5, promising a good separation of CL-A and CL-B. The settling time of this solvent system was 28 s, which is within an acceptable range. Therefore, we selected a solvent system of *n*-butanol–40 m*M* TFA aqueous solution (1:1, v/v) for the HSCCC separation of CL components.

Using the above solvent system, a 20 mg quantity of commercial CL was separated by HSCCC. The retention of stationary phase was 45%. The elution curve monitored at 220 nm is shown in Fig. 17. According to the results of HPLC analysis and the elution curve, the collected effluent was combined into 5 large fractions as shown in Fig. 17. The yields of CL-A and CL-B were 9 mg each and other minor components were 0.5–1.0 mg. HPLC analysis was performed for each fraction. As shown in Fig. 18, the

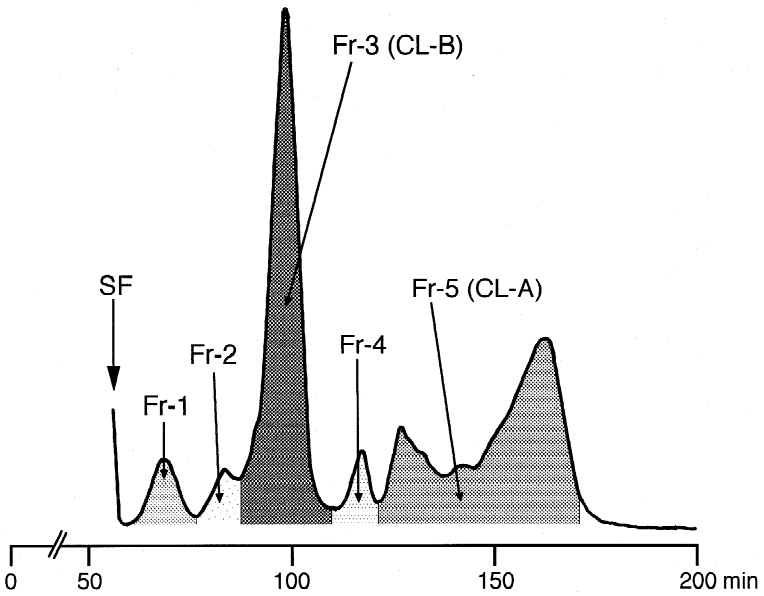


Fig. 17. HSCCC elution curve of commercial CL. Conditions: apparatus: HSCCC-1A, revolution: 800 rpm, solvent system: *n*-butanol–0.04 *M* TFA aq. soln (1:1, v/v), mobile phase: lower phase, flow rate of mobile phase: 2.0 ml/min, detection: 220 nm; sample size: 20 mg.

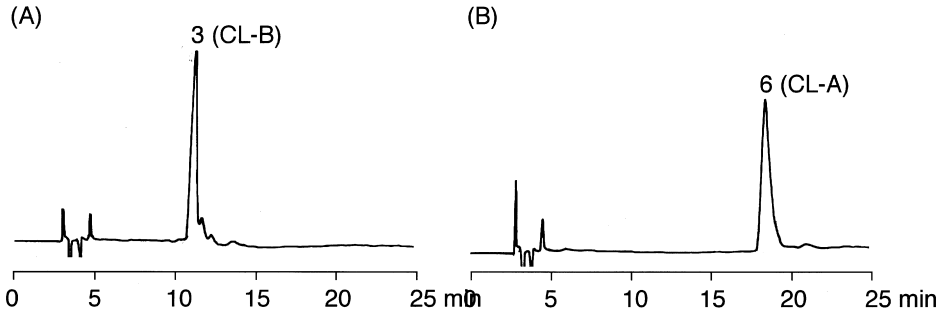


Fig. 18. HPLC profiles of isolated components of CL. (A) CL-A (Fr-5). (B) CL-B (Fr-3).

fractions of CL-A and CL-B showed an almost single peak, each estimated as over 90% pure.

5. Conclusion

Because of the support-free partition system, CCC provides a great advantage over other chromatographic methods by eliminating various complications such as adsorptive loss and deactivation of samples and contamination. As shown by our examples, CCC can isolate various components from a complex mixture of antibiotics by carefully selecting the two-phase solvent system to optimize the partition coefficient (K) of the aimed component(s). The K value for each component can be obtained from a simple test tube partition experiment combined with HPLC analysis of each phase.

Compared with CCD and counter-current extraction, CCC can yield higher partition efficiencies in a shorter elution time. The CCC system can also be applied to microanalytical-scale separations without excessive dilution of samples.

We believe that CCC is an ideal method for purification of antibiotics.

References

[1] Y. Ito, R.L. Bowman, Science 8 (1970) 315.
[2] L.C. Craig, D. Craig, in: A. Weissberger (Ed.), Technique of Organic Chemistry, 2nd ed., Vol. 3, Interscience, New York, 1956, p. 149.
[3] Y. Ito, J. Biochem. Biophys. Methods 5 (1981) 105.
[4] N.B. Mandava, Y. Ito (Eds.), Counter-current Chromatography: Theory and Practice (Chromatographic Science Series, vol. 44), Marcel Dekker, New York, 1988.
[5] W.D. Conway, Counter-current Chromatography, Apparatus, Theory and Applications, VCH, New York, 1990.
[6] Y. Ito, in: E. Heftmann (Ed.), Chromatography, 5th ed., Part A (Journal of Chromatography Library, Vol. 51A), Elsevier, Amsterdam, 1992, Ch. 2, pp. A69–A107.
[7] A.P. Foucault (Editor), Centrifugal Partition Chromatography (Chromatographic Science Series, Vol. 68), Marcel Dekker, New York, 1994.
[8] W.D. Conway, R.J. Petroski (Eds.) Modern Counter-current Chromatography (ACS Symposium Series, No. 593), American Chemical Society, Washington, DC, 1995.
[9] Y. Ito, W.D. Conway (Eds.), High-Speed Counter-Current Chromatography (Chemical Analysis Series, Vol. 132), Wiley-Interscience, New York, 1996.
[10] T. Tanimura, J.J. Pisano, Y. Ito, R.L. Bowman, Science 169 (1970) 54.
[11] K. Okamoto, H. Yonezawa, N. Izumiya, J. Chromatogr. 92 (1974) 147.
[12] K. Hostettmann, C. Appolonia, B. Domon, M. Hostettmann, J. Liq. Chromatogr. 7 (1984) 231.
[13] Y. Ito, R.L. Bowman, Anal. Biochem. 82 (1977) 63.
[14] Y. Ito, R.L. Bowman, J. Chromatogr. 147 (1978) 221.
[15] T.-Y. Zhang, J. Chromatogr. 315 (1984) 287.
[16] Y. Ito, J. Chromatogr. 214 (1981) 122.
[17] Y. Ito, J.L. Sandlin, W.G. Bowers, J. Chromatogr. 244 (1982) 247.
[18] Y. Ito, Adv. Chromatogr. 24 (1984) 181–226.
[19] Y. Ito, CRC Crit. Rev. Anal. Chem. 17 (1986) 65.
[20] A. Berthod, J. Chromatogr. 550 (1991) 677.
[21] Y. Ito, in: Y. Ito, W.D. Conway (Eds.), High-Speed Counter-Current Chromatography (Chemical Analysis Series, Vol. 132) Wiley-Interscience, 1996, Ch. 6, pp. 121–175.
[22] H. Oka, F. Oka, Y. Ito, J. Chromatogr. 479 (1989) 53.
[23] Y. Ito, H. Oka, J.L. Slemp, J. Chromatogr. 475 (1989) 219.
[24] Y. Ito, E. Kitazume, J.L. Slemp, J. Chromatogr. 548 (1991) 81.
[25] Y. Ito, J. Liq. Chromatogr. 8 (1985) 2131.
[26] Y. Ito, Sep. Sci. Technol. 22 (1987) 1971.
[27] Y. Ito, Sep. Sci. Technol. 22 (1987) 1989.

[28] Y. Ito, T.-Y. Zhang, J. Chromatogr. 449 (1988) 135.
[29] Y. Ito, H. Oka, J.L. Slemp, J. Chromatogr. 463 (1989) 305.
[30] M. Bhatnagar, H. Oka, Y. Ito, J. Chromatogr. 463 (1989) 317.
[31] J.-M. Menet, D. Thíbaut, R. Rosset, J. Chromatogr. A 659 (1994) 3.
[32] F. Oka, H. Oka, Y. Ito, J. Chromatogr. 538 (1991) 99.
[33] I.A. Sutherland, J.S. Lee, D.J. Gauvreau, Anal. Biochem. 89 (1978) 213.
[34] H. Nakazawa, P.A. Andrews, N.R. Bachur, Y. Ito, J. Chromatogr. 205 (1981) 482.
[35] H. Nakazawa, C.E. Riggs Jr., M.J. Egorin, S.M. Redwood, N.R. Bachur, R. Bhatnagar, Y. Ito, J. Chromatogr. 307 (1984) 323.
[36] G.M. Brill, J.B. McAlpine, J.E. Hochlowski, J. Liq. Chromatogr. 8 (1985) 2259.
[37] D.G. Martin, R.E. Peltonen, J.W. Nielsen, J. Antibiotics 39 (1986) 721.
[38] I.A. Sutherland, D. Heywood-Waddington, Y. Ito, J. Chromatogr. 384 (1987) 197.
[39] R.H. Chen, J.E. Hochlowski, J.B. McAlpine, R.R. Rasmussen, J. Liq. Chromatogr. 11 (1988) 191.
[40] H. Oka, K.-I. Harada, M. Suzuki, H. Nakazawa, Y. Ito, J. Chromatogr. 482 (1989) 197.
[41] K.-I. Harada, I. Kimura, A. Yoshikawa, M. Suzuki, H. Nakazawa, S. Hattori, K. Komori, Y. Ito, J. Liq. Chromatogr. 13 (1990) 2373.
[42] H. Oka, K.-I. Harada, M. Suzuki, H. Nakazawa, Y. Ito, J. Chromatogr. 538 (1991) 213.
[43] K.-I. Harada, Y. Ikai, Y. Yamazaki, H. Oka, M. Suzuki, H. Nakazawa, Y. Ito, J. Chromatogr. 538 (1991) 203.
[44] H. Oka, Y. Ikai, N. Kawamura, J. Hayakawa, K.-I. Harada, H. Murata, M. Suzuki, Y. Ito, Anal. Chem. 63 (1991) 2861.
[45] S. Drogue, M.-C. Rolet, D. Thiébaut, R. Rosset, J. Chromatogr. 593 (1992) 363.
[46] H. Oka, Y. Ikai, J. Hayakawa, K.-I. Harada, M. Suzuki, A. Shimizu, T. Hayashi, K. Takeba, H. Nakazawa, Y. Ito, J. Chromatogr. A 723 (1996) 61.
[47] Y. Ikai, H. Oka, J. Hayakawa, N. Kawamura, K.-I. Harada, M. Suzuki, H. Nakazawa, Y. Ito, J. Liq. Chromatogr., in press.
[48] R.R. Rasmussen, M.E. Nuss, M.H. Scherr, S.L. Mueller, J.B. McAlpine, L.A. Mitscher, J. Antibiotics 39 (1986) 1515.
[49] L. Bassi, B. Joose, P. Gassmann, H.-P. Kaiser, H. Leuenberger, W. Keller-Schierlein, Helv. Chim. Acta 66 (1983) 92.
[50] R.R. Rasmussen, M.H. Scherr, D.N. Whittern, A.M. Buko, J.B. McAlpine, J. Antibiotics 40 (1987) 1383.
[51] J.E. Hochlowski, M.M. Mullally, G.M. Brill, D.N. Whittern, A.B. Buko, P. Hill, J.B. McAlpine, J. Antibiotics 44 (1991) 1318.
[52] T. Okuda, Y. Ito, T. Yamaguchi, T. Furumai, M. Suzuki, M. Tsuruoka, J. Antibiotics, Ser. A 19 (1966) 85.
[53] G.T. Barry, J.D. Gregory, L.C. Craig, J. Biol. Chem. 175 (1948) 485.
[54] FAO Food and Agriculture Nutrition Paper 41/3, Residue of Some Veterinary Drugs in Animals and Foods, Food and Agriculture Organization of the United Nation, 1990, Ivermectin, pp. 45–64.
[55] Y. Koyama, A. Kurosawa, A. Tsuchiya, K. Takakuda, J. Antibiotics 3 (1950) 457.