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Separation of ivermectin components by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) has been successfully applied to the separation of the ivermectin components. A 25-mg quantity of the sample was separated using a two-phase solvent system of *n*-hexane–ethyl acetate–methanol–water (19:1:10:10, v/v). The fractions were analyzed by high-performance liquid chromatography, nuclear magnetic resonance, and fast-atom bombardment mass spectrometry. The separation yielded 18.7 mg of 99.9% pure ivermectin B1a, 1.0 mg of 96.0% pure ivermectin B1b, and 0.3 mg of 98.0% pure avermectin Bla (precursor of ivermectin).

1. Introduction

The ivermectins B1 are broad-spectrum antiparasitic agents widely used for food-producing animals such as cattle, swine, and horse [1]. They are derived from the avermectins B1, natural fermentation products of *Streptomyces avermitilis* [2]. The avermectins B1 have double bonds between the carbon atoms at positions 22 and 23, whereas the ivermectins B1 have single bonds in these positions (Fig. 1). The ivermectins B1 are a mixture of two major homologs, ivermectin B1a (>80%) and ivermectin B1b (<20%) [1], but the raw isolate also contains various minor components (Fig. 2A). Since effective dosage levels of the ivermectins are very low in all species, a sensitive and specific analytical method is necessary for food sanitation and metabolic studies. To develop these methods highly pure ivermectin components are needed, however, such reference standards are not commercially available and at present a mixture of the components is sold only as a glycerol formulation.

During the past decade high-speed counter-current chromatography (HSCCC) [3] has been

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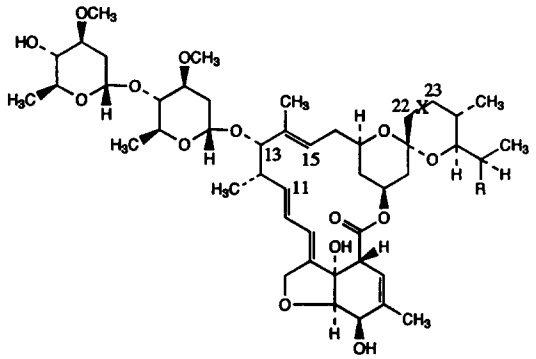

	R	-C_{22}-X-C_{23}-	M.W.
Ivermectin B1a	C_2H_5	-CH_2-CH_2-	874
Ivermectin B1b	CH_3	-CH_2-CH_2-	860
Avermectin B1a	C_2H_5	-CH=CH-	872
Avermectin B1b	CH_3	-CH=CH-	858

Fig. 1. Structures of ivermectins and avermectins.

increasingly used for the separation of various natural products including antibiotics [4–7]. In this study, three ivermectin components were purified using HSCCC. Each component was identified using high-performance liquid chromatography (HPLC), fast-atom bombardment mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR).

2. Experimental

2.1. Reagents

Acetonitrile, *n*-hexane, ethyl acetate, methanol, and *m*-nitrobenzyl alcohol were of analytical grade and purchased from Wako (Osaka, Japan). Ivermectin injection (Ivomec injection, 10 mg/

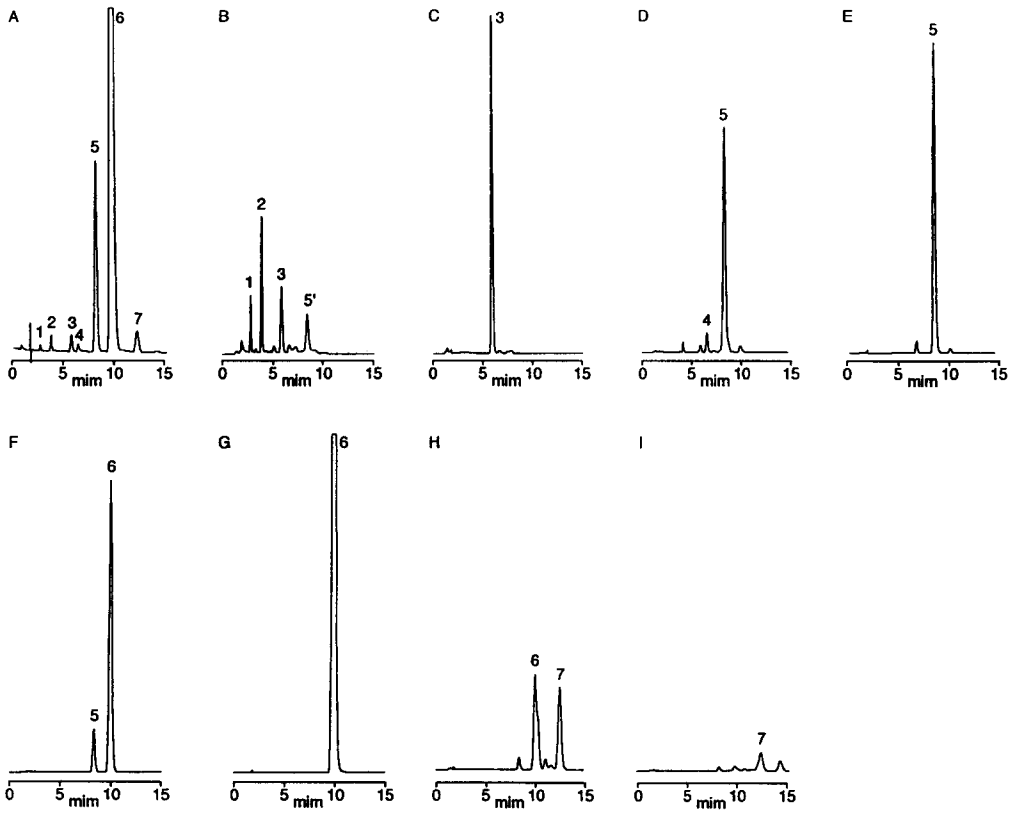

Fig. 2. HPLC separation of the components of ivermectin. (A) Crude ivermectin, (B) fraction I, (C) fraction II, (D) fraction III, (E) fraction IV, (F) fraction V, (G) fraction VI, (H) fraction VII, (I) fraction VIII.

ml in propylene glycol) was purchased from Shionogi (Osaka, Japan).

2.2. Preparation of crude ivermectin

Distilled water (90 ml) was added to ivermectin injection (10 ml), the precipitated crude ivermectin was collected by filtration, and the material on the filter paper was washed twice with distilled water (10 ml). After drying in air, 92.6 mg of crude ivermectin was obtained.

2.3. HPLC analysis

A chromatograph, equipped with a constant-flow pump (LC-100P, Yokogawa, Tokyo, Japan), was used with a variable-wavelength UV–Vis detector (LC-100U, Yokogawa) operated at 245 nm. The separation was performed on TSK GEL-80 Ts ODS (5 μm, 150 × 4.6 mm I.D., TOSO, Tokyo, Japan) with methanol–water (9:1) as the mobile phase at a flow-rate of 1.0 ml/min.

2.4. Measurement of partition coefficient

Approximately 1 mg of the test sample was weighed in a 10-ml test tube to which 2 ml of each phase of the equilibrated two-phase solvent system was added. The tube was stoppered and shaken vigorously for 1 min to equilibrate the sample thoroughly with the two phases. Then, equal volumes of each phase were analyzed by HPLC to obtain the partition coefficients.

2.5. HSCCC separation

The apparatus used was a HSCCC-1A prototype multi-layer coil planet centrifuge (Shimadzu, Kyoto, Japan) with a 10-cm orbital radius which produces a synchronous planetary motion at 800 rpm. The multi-layer coil was prepared by winding a ca. 160 m length of PTFE tubing onto the column holder with a 10-cm hub diameter and a 15-cm hub length, making six coiled layers with a total capacity of about 300 ml. The two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water solution (19:1:10:10) was thoroughly equilibrated in a separatory funnel by repeated vigorous shaking and degassing at room temperature. The column was first entirely filled with the upper non-aqueous stationary phase, then 25 mg of the sample dissolved in 1 ml of each phase was loaded. The column was rotated at 800 rpm, while the lower aqueous mobile phase was pumped into the head of the column (the head–tail relationship of the rotating coil is conventionally defined by the Archimedean screw force, where all objects of different densities are driven toward the head of the coil) at a flow-rate of 2 ml/min by a HPLC pump (LC-6A, Shimadzu). The effluent from the outlet of the column was continuously monitored with a UV detector (SPD-6A, Shimadzu) at 245 nm and then fractionated into test tubes at 2 ml per tube using a fraction collector (DF-2000, Tokyo Rikakikai, Tokyo, Japan).

When the separation was completed, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing them out of the column with pressurized nitrogen gas under slow coil rotation in the tail-to-head elution mode.

2.6. FAB-MS analysis

The FAB mass spectra were obtained with a double-focusing mass spectrometer (JMS-AX505W, JEOL, Tokyo, Japan). A xenon ion gun was operated at 3 kV. The matrix used was *m*-nitrobenzyl alcohol.

2.7. NMR spectral analysis

^{1}H and ^{13}C NMR spectra were recorded on a NMR spectrometer (JNM-A400, JEOL). ^{1}H and ^{13}C Chemical shifts were referenced to residual $CHCl_3$ (7.24 ppm) and C^2HCl_3 (77.0 ppm), respectively. Samples were dissolved in deuterated chloroform (C^2HCl_3).

3. Results and discussion

3.1. Selection of two-phase solvent system

Successful separation by HSCCC depends upon the selection of a suitable solvent system which requires the following considerations [8–10]: (1) For satisfactory retention of the stationary phase, the settling time of the solvent systems should be considerably shorter than 30 s; (2) to avoid excessive waste of solvent, the mixture should provide nearly equal volumes of each phase; and (3) for efficient separation, the partition coefficient (K) of the target compound should be close to 1 and the separation factor between two components ($\alpha = K_2/K_1$, $K_2 > K_1$) should be larger than 1.5. In general smaller K-values may result in a loss of peak resolution, while larger K-values tend to excessively broaden the sample band. A minimum α-value of 1.5 is required for baseline separation in a semi-preparative HSCCC equipment providing moderate partition efficiency around 800 theoretical plates. The K-value of a pure compound can be determined simply by measuring its UV absorbance in both phases after partitioning between candidate phases. When the compounds to be separated are not available in a pure form, as in the present case, their K-values obviously can not be determined by the above method. In this case the following HPLC method can be used: as shown in Fig. 2A, HPLC can separate ivermectin components into seven peaks where the desired components corresponding to peaks 5 and 6 are the ivermectins B1b and B1a, respectively. After partitioning the sample between the two candidate phases, aliquots of the upper and lower zones were analyzed by HPLC. From these two chromatograms the K-value of each component was determined by comparing the peak heights (or areas) between the corresponding peaks.

We have selected a two-phase solvent system composed of *n*-hexane, ethyl acetate, methanol, and water, because it is useful for the separation of components with a broad range of hydrophobicity by modifying the volume ratio of the four solvents [8]. In the *n*-hexane–ethyl acetate–methanol–water (8:2:5:5) 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. This indicated that the component of peak 6 (ivermectin B1a) is mostly partitioned in the upper aqueous phase (Table 1). Although the *n*-hexane–ethyl acetate–methanol–water (9:1:5:5) system gave an improved K-value for peak 6, its K-value was still too large and the α-value (separation factor or ratio of partition coefficients between two analytes) between peaks 6 and 7 is smaller than 1.5. Finally, a slightly less polar solvent mixture of *n*-hexane–ethyl acetate–methanol–water (19:1:10:10) system yielded the best K-values, as indicated in Table 1. In this solvent system, peaks 1 and 2 will be eluted together near the solvent front whereas peak 7 may mostly remain in the column contents. All other peaks with α-values over 1.5 will be completely resolved and elute in

Table 1
Partition coefficients (K) of the components of ivermectin

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.

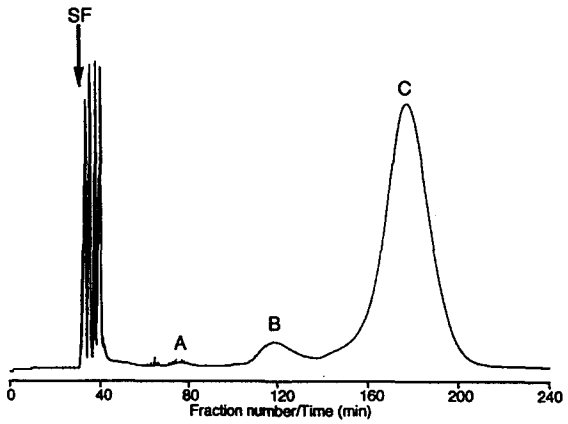

Fig. 3. Separation of crude ivermectin by HSCCC. SF = solvent front.

the order 3, 4, 5, and 6. The settling time of this solvent system was 7 s and the volume ratio of the upper and lower phases was 0.98. Therefore, this solvent system was selected for the separation of ivermectin components.

3.2. Separation of the ivermectin components by HSCCC

A 25-mg amount of crude ivermectin was separated using the above solvent system. The retention of the stationary phase was 67.6%. The total separation time was 4.0 h with a total elution volume of 480 ml.

The HSCCC elution curve of the ivermectin components monitored at 245 nm is shown in Fig. 3 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 1 and 2 (Fig. 2A) correspond to the HSCCC peaks eluted near the solvent front,

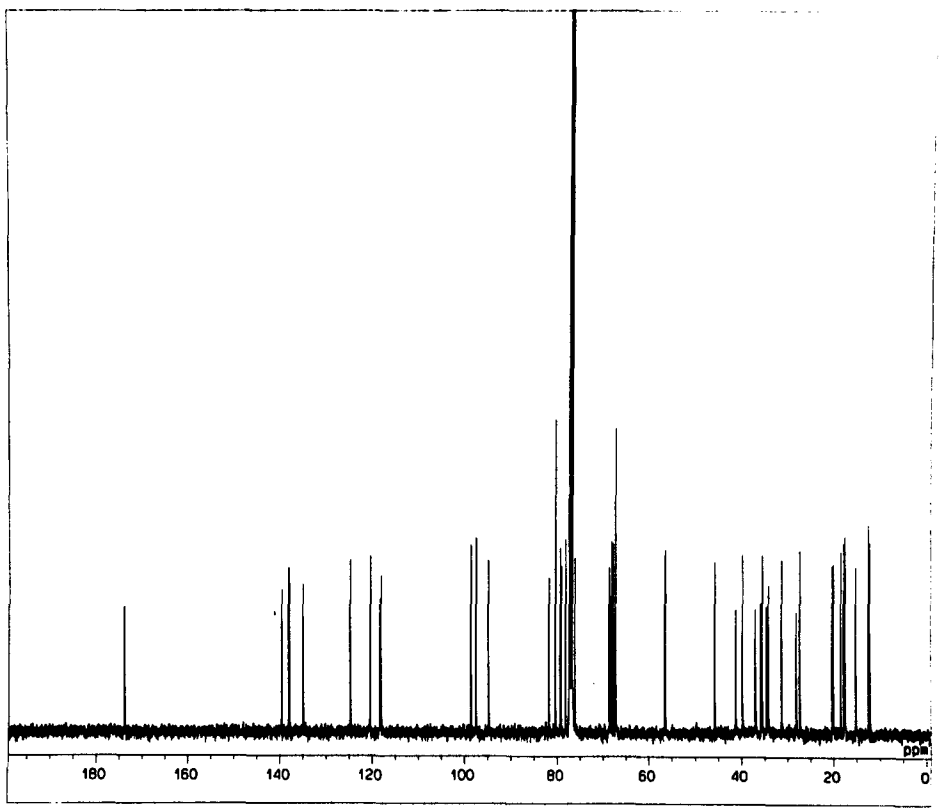

Fig. 4. ^{13}C NMR spectrum (C^2HCl_3) of peak 6.

HPLC peaks 3, 5, and 6 correspond to HSCCC peaks A, B, and C, respectively, and HPLC peak 7 was still retained on the CCC column.

Based on the above HPLC analysis, all collected fractions and column contents were combined into eight pooled fractions (I–VIII). Figs. 2B–I show the HPLC analyses of these combined fractions indicating that fraction II (tube nos. 69–78, 0.3 mg), fraction IV (tube nos. 104–116, 1.0 mg) and fraction Vl (tube nos. 150–180, 18.7 mg) contain almost pure components corresponding to HPLC peaks 3, 5, and 6, respectively, whereas fractions I (tube nos. 31–68, 0.1 mg), III (tube nos. 79–103, 0.4 mg), V (tube nos. 117–149, 3.9 mg), VII (tube nos. 181–249, 0.7 mg), and column contents (1.2 mg) contained multiple components.

In the HPLC analyses of the original sample (Fig. 2A), peaks 3, 5, and 6 constituted about 0.6, 6.8, and 89.9% of the total peak area at 245 nm, respectively. After only a one-step HSCCC operation, the purity of above three components was increased to over 98.0% (Fig. 2C), 96.0% (Fig. 2E) and 99.9% (Fig. 2G), respectively. These results show the high resolving power of HSCCC when careful attention is paid to selection of the proper solvent system to achieve a specific end.

3.3. Identification of the ivermectin components by NMR and FAB-MS

Although no NMR spectra could be measured for peaks 5 and 3 due to the limited amount of samples, 48 signals including three overlapping signals appear clearly in the ^{13}C NMR spectrum of the main component, peak 6 (Fig. 4). They are completely identical with those for ivermectin B1a. In addition, the ^{1}H NMR spectrum of peak 6 (Fig. 5) also coincides with that of

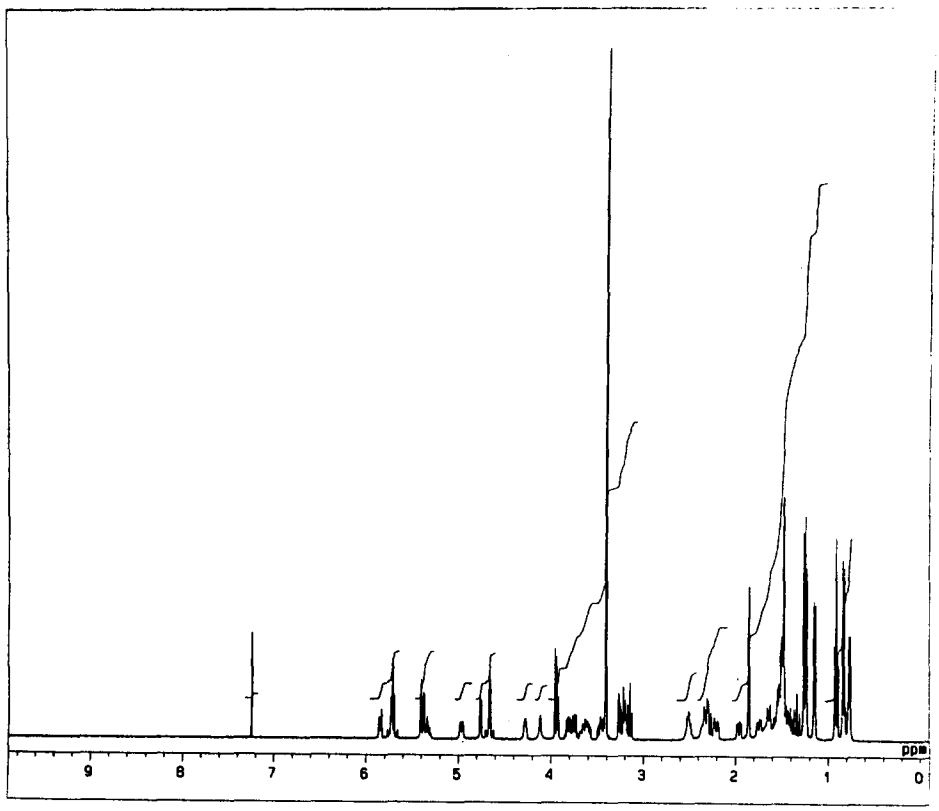

Fig. 5. ^{1}H NMR spectrum ($C^{2}HCl_{3}$) of peak 6.

ivermectin B1a except for the chemical shifts of hydroxyl groups [11]. The FAB mass spectra of fractions VI, IV, and II corresponding to peaks 6, 5, and 3, respectively, are shown in Fig. 6A–C. In the spectrum of fraction VI, the sodium adduct ion of ivermectin B1a is observed at m/z 897 and the molecular mass is determined to be 874. The characteristic fragment ions are at m/z 551, 569, 591, and 607, indicating loss of the sugar moieties.

Figs. 6B and C show spectra of the compounds in fractions IV and II where the sodium adduct ions at m/z 883 and 895 show the molecular masses to be 860 and 872. Loss of the disaccharide moieties again gives the aglycone-derived ions at m/z 537–605, indicating that the sugar moieties are identical in all fractions. Therefore, these results indicate that the compounds in fractions IV and II are ivermectin B1b and avermectin B1a (precursor of ivermectin), respectively.

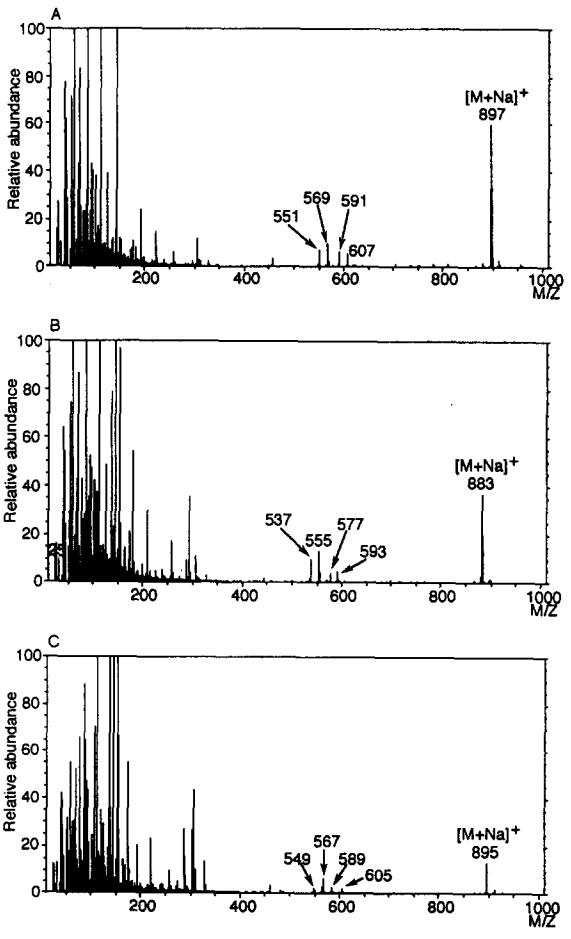

Fig. 6. FAB mass spectra of the components from crude ivermectin. (A) Fraction VI, (B) fraction VII, (C) fraction VIII.

4. Conclusion

Using HSCCC we were able to purify three components of ivermectin with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (19:1:10:10). From 25 mg of the crude sample, we obtained 18.7 mg of 99.9% pure ivermectin B1a, 1.0 mg of 96.0% pure ivermectin B1b, and 0.3 mg of 98.0% pure avermectin B1a, one component of avermectin B1 (precursor of ivermectin). The overall results of our studies indicate that HSCCC is a powerful technique for the purification of ivermectin components.

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