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Journal of Chromatography A, 903 (2000) 93–98

JOURNAL OF CHROMATOGRAPHY A

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Separation of spiramycin components using high-speed counter-current chromatography

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Received 19 June 2000; received in revised form 31 August 2000; accepted 31 August 2000

Abstract

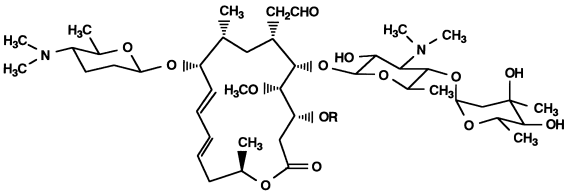
High-speed counter-current chromatography was successfully applied to the separation of the main components of spiramycin. A 25-mg quantity of sample was separated using a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:6:5:5), and the fractions were analyzed by high-performance liquid chromatography and fast atom bombardment mass spectrometry. The separation yielded 13.4, 0.7 and 1.7 mg of spiramycins I, II, and III with purities of 98.2, 92.3 and 97.4%, respectively.

Keywords: Counter-current chromatography; Spiramycins; Macrolide antibiotics; Antibiotics

1. Introduction

Spiramycin (SPM) is a 16-membered macrolide antibiotic widely used for livestock such as cattle, swine and poultry [1]. It is produced by *Streptomyces ambofaciens* and consists of multiple components including SPM I, SPM II and SPM III (Fig. 1) [2]. The quantity of each component varies according to the manufacturer. Since residues of the drug in tissues of the livestock are highly undesirable, a reliable analysis method should be used to inspect the residues for food sanitation. The development of the methods requires pure SPM components, however, such reference standards are not commercially available.

During the past decade high-speed counter-current chromatography (HSCCC) [3] has been increasingly used for the separation of various natural products



	R	MW
Spiramycin I	— H	842
Spiramycin II	— $COCH_3$	884
Spiramycin III	— $COCH_2CH_3$	898

Fig. 1. Structures of spiramycins.

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00903-1

including antibiotics [4–8]. In this study, three SPM components were purified using HSCCC. Each component was identified using high-performance liquid chromatography (HPLC) and fast atom bombardment mass spectrometry (FAB-MS).

2. Experimental

2.1. Reagents

Acetonitrile, *n*-hexane, ethyl acetate, methanol, phosphoric acid, and glycerol were of analytical grade and purchased from Wako (Osaka, Japan). Spiramycin preparation was purchased from Kyowa Hakko Kogyo (Tokyo, Japan).

2.2. HPLC analysis

A chromatograph, equipped with a constant-flow pump (PU-970, Jasco, Tokyo, Japan), was used with a variable-wavelength UV–Vis detector (UV-970, Jasco) operated at 232 nm. The separation was performed on a Cosmosil $5C_{18}$ AR-II (5 μm, 150× 4.6 mm I.D., Nacarai, Tokyo, Japan) column with acetonitrile–0.05 *M* phosphoric acid (pH 3.0) (2:8) as the mobile phase at a flow-rate of 1.0 ml/min.

2.3. 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.

2.4. 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 (3:6:5:5) solution was thoroughly equilibrated in a separatory funnel by repeating vigorous shaking three times each followed by disposal of the generated gas by inverting the vessel and manipulating its stopcock 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 with different densities, either lighter or heavier than the surrounding medium, are driven toward the head of the coil) at a flow-rate of 1 ml/min by a HPLC pump (LC-6A, Shimadzu). The effluent from the outlet of the column was fractionated into test tubes at 1 ml per tube using a fraction collector (DF-2000, Tokyo Rikakikai, Tokyo, Japan).

After 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.

2.5. FAB-MS analysis

The FAB mass spectra were obtained with a double-focusing mass spectrometer (JMS-HX110, JEOL, Tokyo, Japan). A xenon ion gun was operated at 10 kV. The matrix used was glycerol.

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 [8–11]: (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

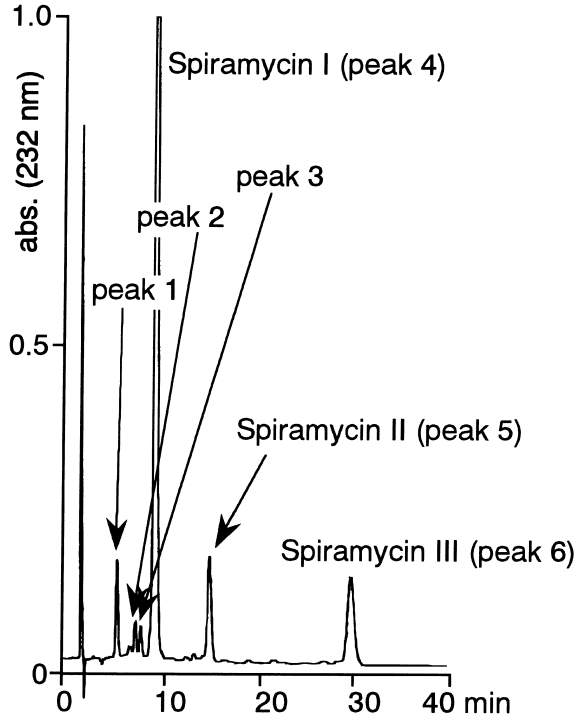

Fig. 2. HPLC separation of spiramycin components in the original sample. HPLC conditions: see Experimental.

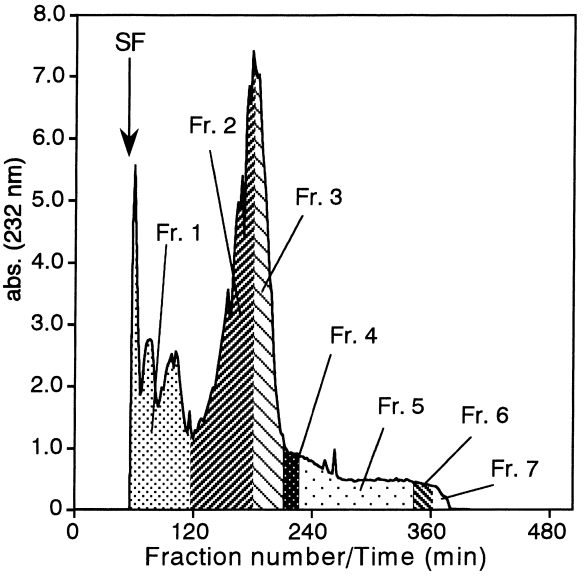

Fig. 3. Separation of spiramycin components by HSCCC. HSCCC conditions: see Experimental. Sample amount: 25 mg. SF= Solvent front.

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 produce excessive sample band broadening. 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 obtained by computing the ratio of its UV absorbance between the two phases. When the compounds to be separated are not available in a pure form, as in the present case, their K values of individual components cannot be determined by the above method. In this case the following HPLC method can be used: after partitioning the sample between the two solvent phases, aliquots of the upper and lower layers were analyzed by HPLC. From these two chromatograms the K value of each component is determined by computing the ratio of the peak heights (or areas) between the corresponding peaks. Fig. 2 shows HPLC separation of spriramycin components in which six peaks

Table 1
Partition coefficients and separation factors (α) of spiramycin components

Solvent systems	Peak No.						
	3		4		5		6
n-hexane–ethyl acetate–methanol–water		(α)		(α)		(α)	
(1:1:1:1)	0.097	(2.87)	0.280	(1.97)	0.550	(2.22)	1.22
(3:7:5:5)	0.375	(2.43)	0.913	(1.22)	1.11	(2.07)	2.31
(3:6:5:5)	0.405	(1.79)	0.725	(1.73)	1.26	(1.71)	2.15
(2:8:5:5)	0.830	(1.16)	0.701	(3.19)	2.27	(1.29)	2.94

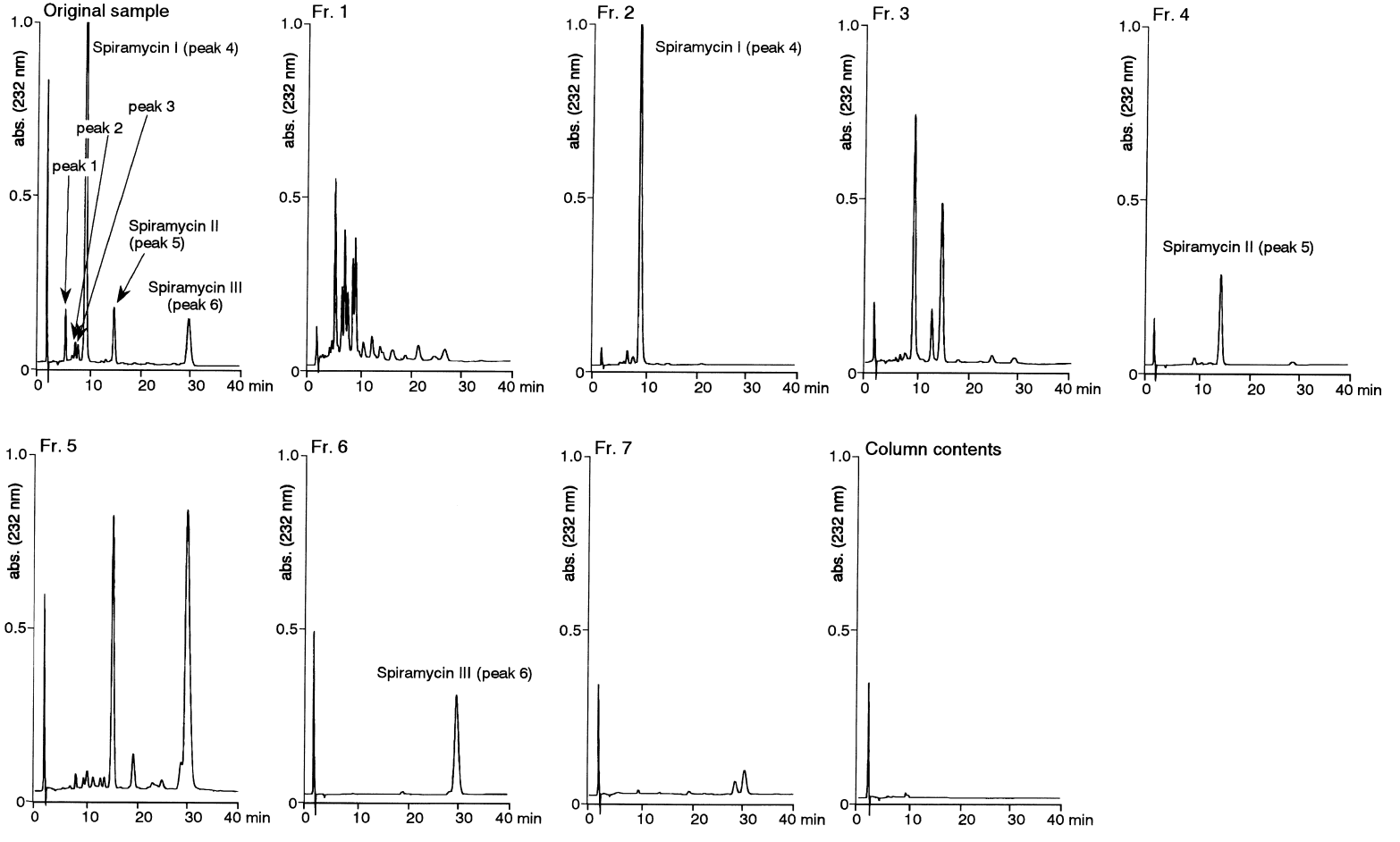

Fig. 4. HPLC separation of spiramycin components. Fraction numbers as in Fig. 3. HPLC conditions: see Experimental. Injection volume: 10 μl.

were resolved including peaks 4, 5 and 6 that correspond to the target compounds of spiramycins I, II and III, respectively.

We have selected a two-phase solvent system composed of *n*-hexane, ethyl acetate, methanol and water, because it provides a broad range of hydrophobicity by modifying the volume ratio of the four solvents [8]. In the *n*-hexane–ethyl acetate–methanol–water (1:1:1:1) system first examined, the *K* values of the components corresponding to peaks 3, 4, 5 and 6 were 0.097, 0.280, 0.550 and 1.22, respectively, indicating that the components of peaks 3 and 4 are mostly partitioned in the lower aqueous phase (Table 1). Although the *n*-hexane–ethyl acetate–methanol–water (3:7:5:5) and (2:8:5:5) systems improved *K* values for these peaks, α values between peaks 4 and 5 in the (3:7:5:5) system and between peaks 3 and 4 in the (2:8:5:5) system were smaller than 1.5. In addition, the *K* value of peak 6 in the (2:8:5:5) system is too large. Finally, a moderately polar solvent mixture of *n*-hexane–ethyl acetate–methanol–water (3:6:5:5) yielded the best *K* values as indicated in Table 1. In this solvent system, all target components show α values of over 1.5 promising complete separation of these compounds to elute in the order of 3, 4, 5 and 6. The settling time of this solvent system was 25 s and the volume ratio of the upper and lower phases 0.98. Therefore, this solvent system was selected for the separation of spiramycin components.

3.2. Separation of spiramycin components by HSCCC

A 25-mg quantity of spiramycin was separated using the above solvent system. The retention of the stationary phase was 78.9%. The total separation time was 6.3 h with a total elution volume of 380 ml. The HSCCC fractions were analyzed by HPLC, and their absorbance was measured at 232 nm to draw the elution curve (Fig. 3). HPLC analysis of each fraction revealed that, as expected, the components eluted in the order of peaks 3, 4, 5 and 6.

Based on the HPLC analysis and the elution curve, all collected fractions were combined into seven pooled fractions (fractions 1–7). Fig. 4 shows the HPLC analysis of these combined fractions: fractions 2 (tube No. 112-177, 13.4 mg), 4 (tube No. 208-223, 0.7 mg), and 6 (tube No. 343-360, 1.7 mg) contained almost pure components corresponding to HPLC peaks 4 (spiramycin I), 5 (spiramycin II), and 6 (spiramycin III), respectively. On the other hand, fractions 1 (tube No. 55-111, 4.1 mg), 3 (tube No. 178-207, 2.1 mg), 5 (tube No. 224-342, 1.1 mg), and 7 (tube No. 361-380, 0.3 mg) contained multiple components.

In the HPLC analysis of the original sample (Fig. 2), peaks 4, 5 and 6 constituted about 79.9, 10.5 and

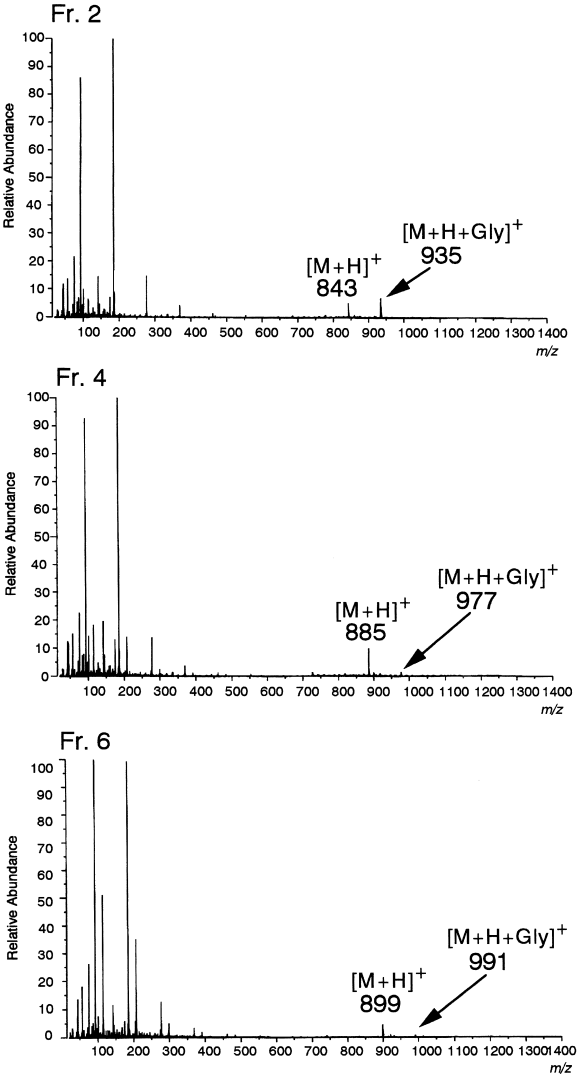

Fig. 5. FAB mass spectra of the isolated components of spiramycin.

8.4% of the total peak area at 232 nm, respectively. After only one-step operation by HSCCC, the purity of above three components was increased to 98.2% (Fig. 4), 92.3% (Fig. 4), and 97.4% (Fig. 4), respectively. These results demonstrate the high resolving power of HSCCC achieved by the careful selection of the proper solvent system.

3.3. Identification of the components of spiramycin by FAB-MS

Fig. 5 shows the FAB mass spectra of fractions 2, 4 and 6 corresponding to peaks 4, 5 and 6, respectively. In these spectra, protonated molecules $[M+H]^+$ and the glycerol adduct ions $[M+H+Gly]^+$ of the isolated components are clearly observed at m/z 843 and 935 for fraction 2, m/z 885 and 977 for fraction 4 and m/z 899 and 991 for fraction 6, respectively. Therefore, these results indicate that the compounds in fractions 2, 4 and 6 are spiramycins I, II and III, respectively.

4. Conclusion

Using HSCCC we were able to purify three components of spiramycin with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:6:5:5). From 25 mg of the sample, we obtained 13.4 mg of 98.2% pure spiramycin I, 0.7 mg of 92.3% pure spiramycin II, and 1.7 mg of 97.4% pure spiramycin III. The overall results of our studies indicated that HSCCC is a powerful technique for the purification of spiramycin components.

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