SEPARATION OF THE FILIPIN COMPLEX BY GRADIENT-ELUTION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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A method has been developed for the separation of the filipin complex components by gradient-elution high performance liquid chromatography (HPLC). The elution order for the major filipin components (filipins I~IV) was established by first isolating the component fractions by thin-layer chromatography. Each component fraction was then subjected to gradient HPLC. The order of elution for the major filipin components was from first to last: III, IV, II and I. The unexpected reversal in the elution order for filipins III and IV may be evidence that the two filipins are stereoisomeric at the C-1′ position. Finally, gradient elution HPLC was used to compare various preparations of filipin. In addition, the technique has been applied to other preparations of polyene antibiotics which have structures similar to that of filipin.

High performance liquid chromatography (HPLC) has been performed previously on the filipin complex (filipins I~IV) by MECHLINSKI and SCHAFFNER[1]. Their results indicated that the complex could be resolved into two major and three minor components. Thin-layer chromatography (TLC) of the complex gave somewhat different results; one major and two minor components. No identification of the components was made with respect to the filipin fractions previously found by BERGY and EBLE[2]. Recently, PANDEY *et al.*[3] have reported on the HPLC of fungichromin in which filipin III was found as a minor component. In addition, they found that the filipin complex contains fungichromin. In both of the studies cited, the solvent systems used for the HPLC separations contained relatively high proportions of water. MECHLINSKI and SCHAFFNER used a system of water - methanol - tetrahydrofuran in a volume ratio of 420: 90: 60. PANDEY *et al.* used a methanol - water system with a ratio of 60: 40.

This study is concerned with the HPLC separation of the filipin complex components (filipin I~IV) by the use of a solvent gradient. Separations were carried out with methanol - water mixtures which contained between 10 and 25% water. Identifications of the major filipin fractions in the HPLC separation of the complex were made on the basis of filipin fractions which were previously isolated by TLC. A comparison is made between the compositions of the filipin complex originally produced by WHITFIELD *et al.*[4] and that produced in our laboratory.

Materials and Methods

Filipin

Filipin was isolated from a culture of *Streptomyces filipinensis* which was the generous gift of A. J. LYONS, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL. The crude filipin was purified by a method similar to the one used by PATTERSON *et al.*[5]. The filipin complex was then stored lyophilized in the dark at −20°C degrees centigrade.

A sample of the original filipin complex was the generous gift of R. L. KEENE, The Upjohn Company, Kalamazoo, Michigan.

Thin-layer Chromatography (TLC)

The TLC separation of filipin was carried out on pre-coated plates (silica gel GHLF, 250 μ Analtech) by the method of BERGY and EBLE[2]. The filipin fractions were removed mechanically, suspended in 2 ml of H_2O and extracted with 1.5 ml of EtOAc. The EtOAc was removed *in vacuo* and the fractions were each dissolved in 50 μl of MeOH. HPLC samples were taken from these solutions.

High Performance Liquid Chromatography (HPLC)

HPLC of the filipin was performed on a Beckman Model 344 Liquid Chromatographic System in conjunction with a Hitachi Model 100-40 variable wavelength ultraviolet-visible detector. The analytical wavelength was 338 nm. The separations were performed on an Altex Ultrasphere-ODS 5 μ column (4.6 mm ID $\times$ 15 cm) with an Altex Ultrasil-ODS pre-column (3.2 mm ID $\times$ 4.5 cm) at a flowrate of 1 ml/minute. The solvents were HPLC grade H_2O and MeOH from J. T. Baker.

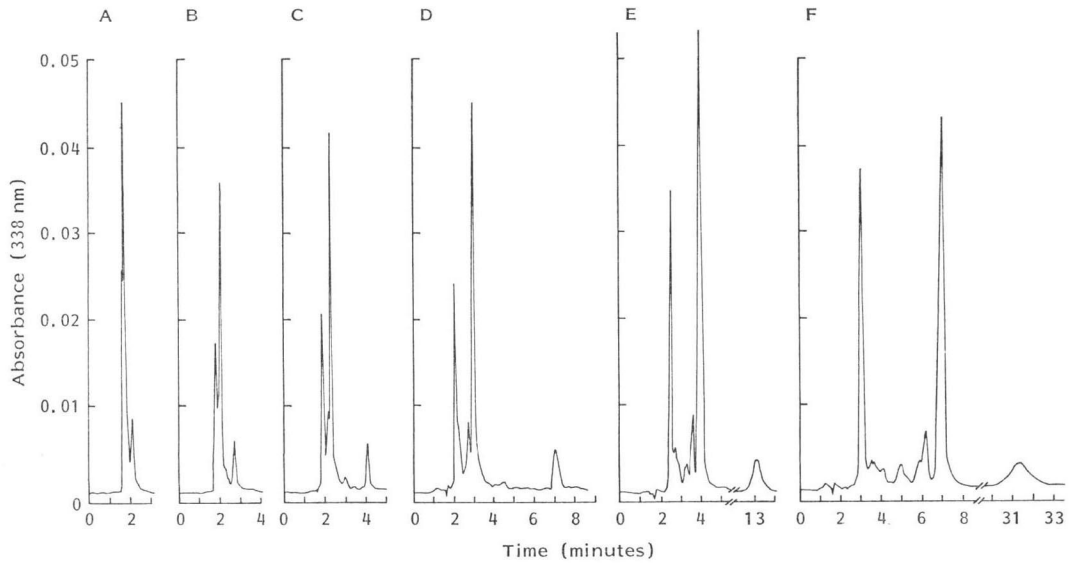
The solvent composition for all gradient runs was maintained at a ratio of 75: 25, MeOH - H_2O for the first 6 minutes after injection. The composition was then changed to 90: 10, MeOH - H_2O over a two minute interval and maintained at that composition for an additional 7 minutes. At 15 minutes after injection, the solvent composition was returned to 75: 25, MeOH - H_2O.

HPLC samples were taken from solutions which were prepared by dissolving filipin in HPLC MeOH.

Results and Discussion

The solubility of filipin in various solvents has been characterized by WHITFIELD *et al.*[4]. Since the filipin complex is soluble in methanol and relatively insoluble in water, it should be a prime candidate for separation by gradient HPLC on a reversed phase column. Fig. 1 shows the results for the non-gradient HPLC separations of the filipin complex with various methanol - water solvent compositions. As expected, the chromatograms exhibit better separations for the major filipin components as the percentage of water is increased. The best overall separations occur at a solvent composition of 85: 15, methanol - water. Further increase in the percentage of water yields better resolution between the first two major components but results in considerable bandspread and an inconveniently long retention time

Fig. 1. Non-gradient HPLC separations of the filipin complex prepared by the authors.
Percentages of H_2O in MeOH were from A through F: 0, 5, 10, 15, 20 and 25, respectively.

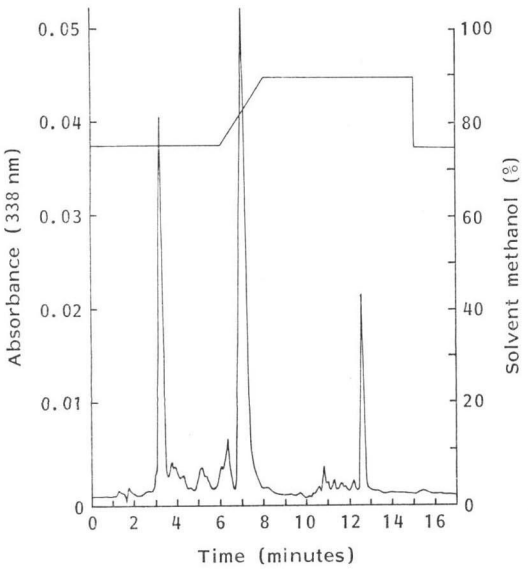


for the last component. In order to overcome these problems, a solvent gradient program was used to achieve optimal separations.

Fig. 2 shows the gradient HPLC separation of the filipin complex prepared in our laboratory. The solvent composition program is shown in the upper portion of that same figure. Besides the 3 main filipin components, there are approximately 25 minor components scattered throughout the chromatogram.

In order to identify the major components in Fig. 2, a sample of the original Upjohn filipin complex was separated into individual components by TLC. As expected, 4 component fractions were found. Each component fraction as well as the complex as a whole was then subjected to gradient HPLC. Figs. 3 and 4 show respectively the results of the separations for both the individual components (already separated by TLC) and for the complex. Filipin I appears to be a mixture of 1 major and 17 minor components. Filipin II is also a mixture and consists of 9 minor and 4 major components with the largest having a retention time of about 7.8 minutes. The chromatogram of the filipin III fraction shows 6 minor and 1 major component while the filipin IV fraction contains a single major component with significant filipin III contamination and two small shoulders on the major peaks. In other separations not shown, the filipin IV component was isolated with almost no filipin III contamination.

Fig. 2. Gradient program and gradient HPLC separation of the filipin complex prepared by the authors.



One of the more striking results of the foregoing identification process is the fact that filipin IV elutes after filipin III. Based on the elution order in TLC, this observation was quite unexpected. This phenomenon may possibly be explained by the isomeric relationship between

Fig. 3. Gradient HPLC separations performed on each of the four TLC fractions (filipins I~IV) isolated from the filipin complex prepared by the Upjohn Co.

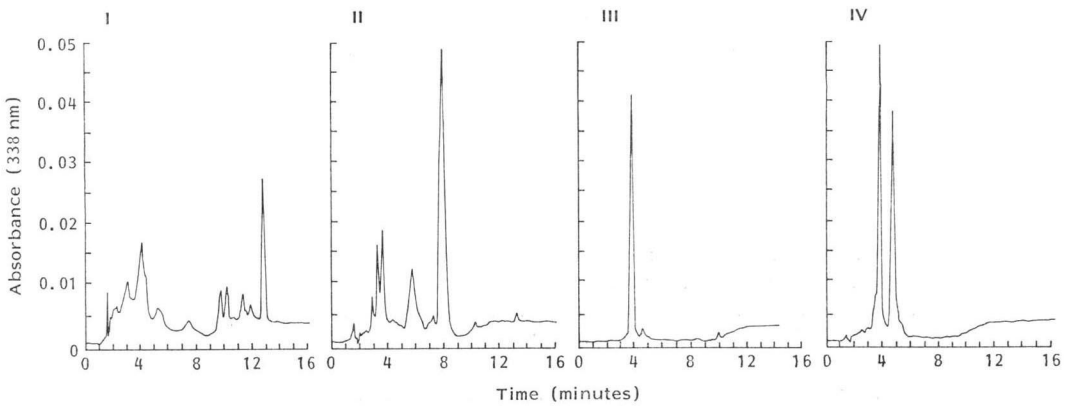


Fig. 4. Gradient HPLC separation of the filipin complex prepared by the Upjohn Co.

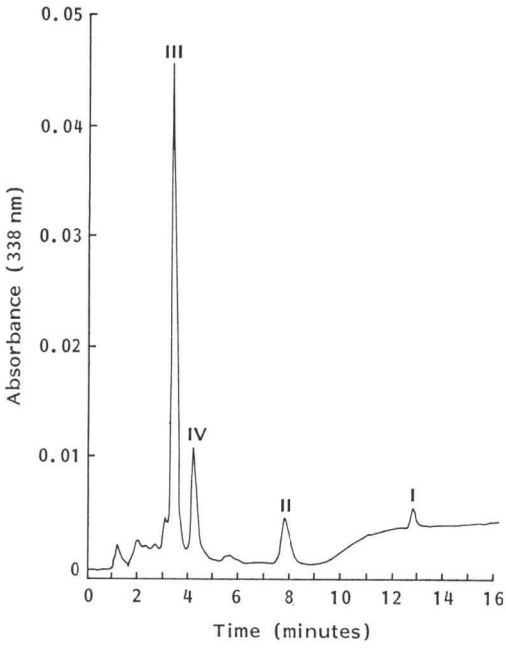
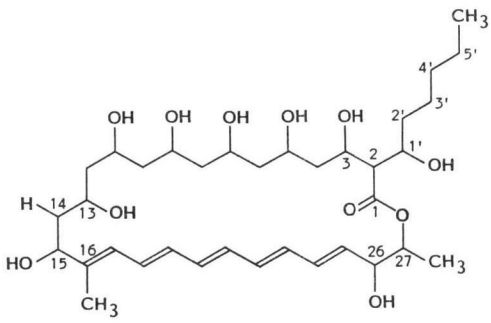


Fig. 5. Structure of filipin III.



the two filipins. In two reports on the structures of filipins III and IV, PANDEY and RINEHART[6] and PANDEY *et al.*[7] have found that the two components are stereoisomeric at the C-3 or C-1′ positions (Fig. 5). Since reverse phase liquid chromatography involves the hydrophobic interaction of the sample molecule with the stationary phase, it might be expected that even slight changes in the orientation of the hydrophobic *n*-hexyl tail of the filipin molecule may have a large effect on its elution time. If such is the case, the experimental results indicate that the two filipin components differ at the C-1′ position.

Another result of the foregoing identification process is the observation that the filipin prepared in our laboratory (Fig. 2) lacks the filipin IV component present in the Upjohn preparation (Fig. 4). Tests on other filipin preparations suggest that the quantity of each filipin component in the complex is affected by the silicic acid purification scheme used in the method of PATTERSON *et al.*[5]. Although all of our other preparations show the presence of filipin IV, there is always a diminution in the amounts of filipins III and IV when compared to those of filipins I and II. This phenomenon appears to be due to some retention of the stereoisomers (filipins III and IV) on the silicic acid column. Our best results were obtained by elution with 85: 15, methylene chloride - methanol instead of the 90: 10 ratio used in the original scheme. However, even with the new ratio there is still some retention of the stereoisomers.

In conclusion, gradient elution HPLC has been shown to be a useful tool for structural elucidation as well as for the comparison of various filipin complex preparations. In addition, this same technique can and has been used successfully (unpublished work) for the comparisons of other polyene antibiotic preparations such as fungichromin[3] and elizabethin[8].

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