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## Note

# Identification and determination of the qualitative composition of nystatin using thin-layer chromatography and high-performance liquid chromatography

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A number of tetraene antifungal antibiotics have been isolated from species of *Streptomyces*; nystatin is an important member of this group. Commercial nystatin has been shown to be a complex mixture of closely related compounds[1], it has been separated into three constituents (designated nystatins $A_1$, $A_2$, $A_3$)[2]. The presence of a heptaene component has been demonstrated by ultraviolet (UV) spectrophotometry[2] and by high-speed liquid chromatography[3]. During the examination of samples of nystatin obtained from different sources it was necessary to compare the composition of the samples. Several thin-layer chromatographic procedures have demonstrated the heterogeneous composition of nystatin[4] and the closely related antibiotic polyfungin[5]. This report describes our experience with the thin-layer chromatographic methods and the development of a high-performance liquid chromatographic method for determining the qualitative composition of nystatin.

## EXPERIMENTAL

### *Materials*

Samples of pharmaceutical grade nystatin available during 1975–1979, and representing all the principle manufacturers from China, Hungary, Italy, U.S.A. and U.S.S.R., were used. All the samples of nystatin (20) and polyfungin (2) complied with the requirements of the British Pharmacopoeia for Nystatin (Dermatalogical)[6]. Samples of amphotericin A, lucensonmycin, natamycin and polyfungin were generously provided by The Squibb Institute (Princeton, U.S.A.), Carlo Erba (Milan, Italy), Gist Brocades (Delft, The Netherlands) and Instytut Przemyslu Farmaceutycznego (Warsaw, Poland), respectively. Analytical grade solvents and reagents were from BDH (Poole, Great Britain).

### *Thin-layer chromatography*

Commercially available precoated silica gel plates 10 × 10 cm (silica gel 60 TLC plates, E. Merck, Darmstadt, G.F.R., and Nano-plates Sil-20 $UV_{254}$, Macherey, Nagel & Co., Düren, G.F.R.) were used. The mobile phases consisted of: method 1, *n*-amyl alcohol–glacial acetic acid–water (2:1:1, v/v/v); method 2, ethyl acetate–propan-2-ol–water (5:5:1, v/v/v); and method 3, lower phase of chloroform–meth-

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anol–water (20:22:10, v/v/v). For each method a saturated developing chamber was used. In method 2 the plates were pretreated by spraying with a solution containing 0.1 *M* phosphoric acid and 0.1 *M* sodium tungstate, pH 6.7, then dried at 100°C for 60 min before use. In method 3 the plates were prerun in the mobile phase, then dried at 100°C for 60 min before use. Solutions (3 mg $ml^{-1}$; lucensomycin, 1.5 mg $ml^{-1}$) were freshly prepared in methanol and 1 to 2 $\mu$l applied to the plates. After development the plates were air dried and visualized either in UV light at 254 nm or after spraying with 10% (v/v) sulphuric acid (sp.gr. 1.84) in ethanol and heating at 100°C until the spots showed.

*High-performance liquid chromatography*

The apparatus consisted of two reciprocating pumps, a gradient controller (Constametric I, Constametric II G and a gradient master, Model 1601, Laboratory Data Control) and a variable wavelength spectrophotometer fitted with 75-$\mu$l flow-through cell. A pre-column (35 × 4 mm I.D.) and a column (100 × 4 mm I.D.) were packed with MOS Hypersil, 5 and 3 $\mu$m, respectively (Shandon Southern Products, Runcorn, Great Britain); other packings tried included Spherisorb ODS, 5 $\mu$m; Spherisorb Hexyl, 5 $\mu$m (Phase Separations, Queensferry, Great Britain) and Nucleosil 5 $C_8$, 5 $\mu$m (Macherey, Nagel & Co.). The mobile phase was composed of methanol and sodium acetate buffer pH 5.8; it was filtered through a glass microfibre filter and degassed prior to use. The gradient used was provided by a starting solution of 55.0% (v/v) methanol in 0.005 *M* acetate buffer and a final solution of 70.0% (v/v) methanol in 0.005 *M* acetate buffer, both mixtures were pH 6.8 ± 0.1. The selected gradient

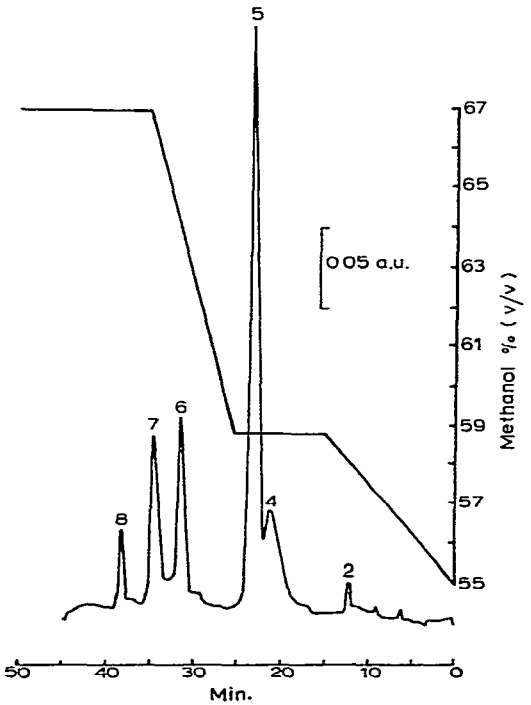


Fig. 1. High-performance liquid chromatogram of polyfungin sample 1, showing gradient profile.

elution profile was a linear increase in methanol concentration from 55.0 to 58.75% (v/v) in 15 min then held for 10 min, then a linear increase from 58.75 to 67.0% (v/v) methanol in 10 min and maintained for a further 15 min (Fig. 1). The column was re-equilibrated with the starting solution in the course of 5 min before injecting the next sample. The flow-rate was 1 ml $min^{-1}$ and the pump outlet pressure was 3200 p.s.i. Samples were dissolved in methanol 3 to 5 mg $ml^{-1}$ and 5-$\mu$l volumes injected on to the column. For the detection of tetranes the eluent was monitored at 304 nm sensitivity 0.5 a.u.f.s., heptanes were monitored at 382 nm sensitivity 0.2 a.u.f.s.

## RESULTS AND DISCUSSION

### *Thin-layer chromatography*

The high-performance plates gave spots which were distinct using methods 1 and 2; both methods showed the complex composition of some nystatin samples and polyfungin. Method 3 gave the best separation and resolution of the antibiotics; it worked equally well with both types of silica plate. The separation was improved when the loaded plates were equilibrated in the saturated developing chamber for 30 min prior to running and when the plates were developed twice. All that was necessary was to air-dry the plates after the first run, then replace the plates in the developing chamber and run for a second time. Typical separations obtained with each method are shown in Fig. 2.

The main component in the majority of the samples of nystatin (13) was taken to be nystatin $A_1$. Nystatin $A_1$, a large-ring tetraene, could be distinguished from the two small-ring tetraenes lucensomycin and natamycin, but it could not be differentiated from the other large-ring tetraene amphotericin A. Seven of the samples of nystatin were shown to be complex mixtures in which nystatin $A_1$ was a minor component. Qualitatively, polyfungin was similar to these samples, though there were quantitative differences.

### *High-performance liquid chromatography*

Typical chromatograms are shown (Figs. 1, 3 and 4), the corresponding principal peaks have been numbered to help in identification. Up to 6 substantial components could be resolved in some of the multicomponent nystatin samples using MOS Hypersil (Fig. 3A), whereas only five could be demonstrated with the other packings tried. MOS Hypersil was the only packing which facilitated the separation of a mixture of nystatin $A_1$ and amphotericin A (Fig. 4A). The optimum overall pH of the mobile phase was found to be between pH 6.5 and 7.0. Reducing the pH increased the retention times; below pH 4 the sample remained on the column. Though increasing the pH above 7.0 reduced the retention times, some components eluted together, *e.g.* peaks 5 and 6. The ionic strength had a greater effect upon the separation of peaks 1 to 5 than upon that of peaks 6 to 8. At a low concentration (0.001 *M*) the retention of the first five principal peaks was low; increasing the molarity to 0.01 *M* increased the retention times, though peaks 3 and 5 combined. Increasing the methanol concentration of the mobile phase reduced the retention times of the last three principle peaks. Eventually a mobile phase of 0.005 *M* acetate buffer with the concentration of methanol increasing from 55.0 to 67.0% (v/v), with an overall pH 6.8 $\pm$ 0.1 was chosen; this allowed the resolution of the principal components found in samples of nystatin and polyfungin within 45 min.

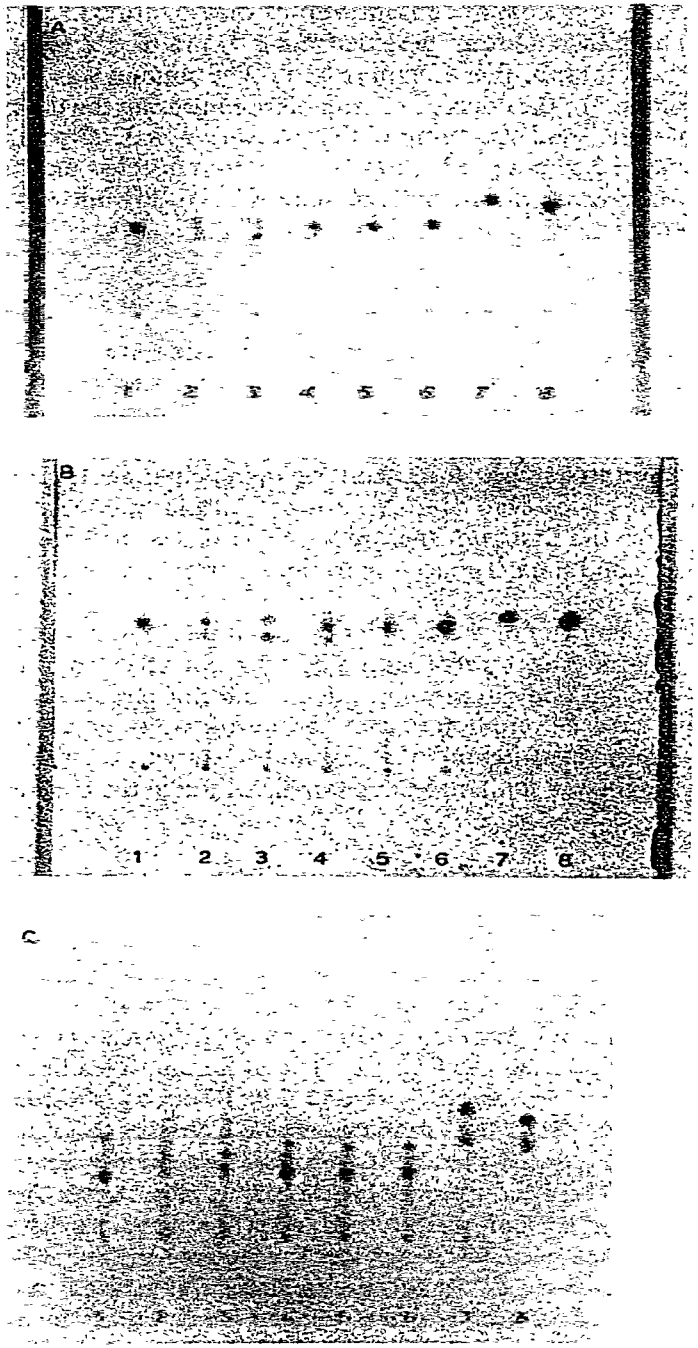


Fig. 2. Thin-layer chromatograms of tetraenes. (A) method 1 using a high-performance plate; (B) method 2 using a high-performance plate; (C) method 3 using a silica gel 60 plate. 1 = nystatin sample 3; 2 = nystatin sample 14; 3 = nystatin sample 18; 4 = polyfungin sample 2; 5 = nystatin sample 1; 6 = amphotericin A; 7 = lucensomycin; 8 = natamycin.

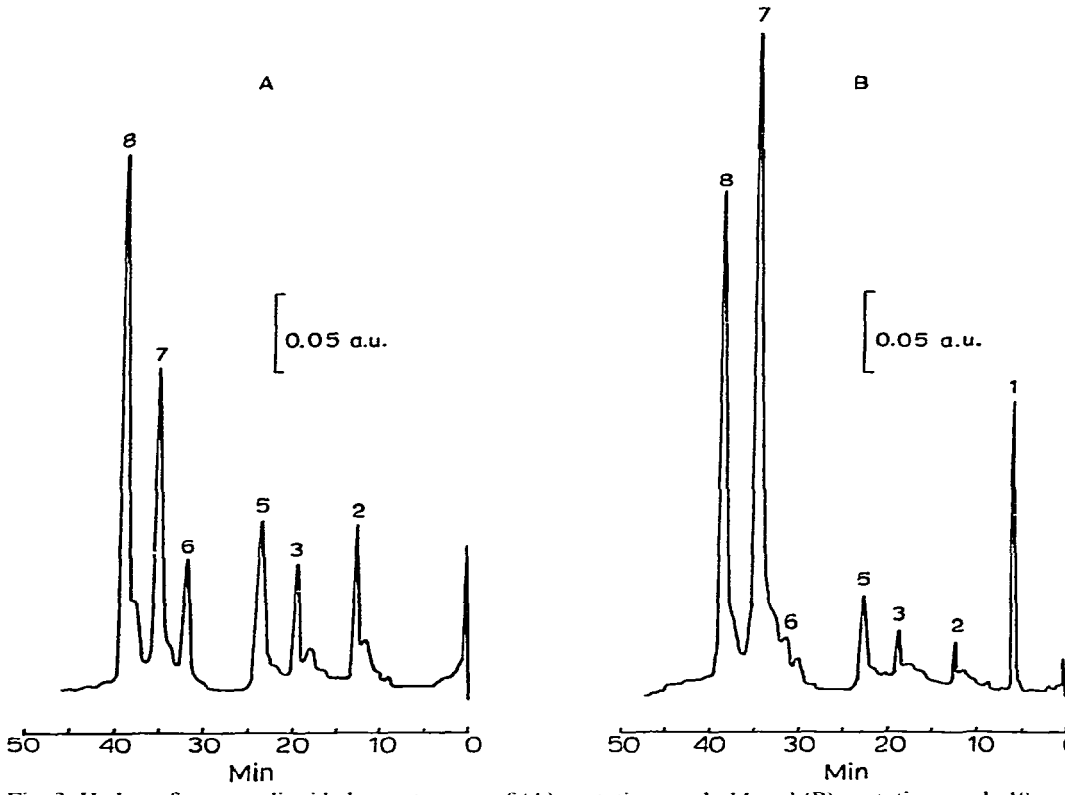


Fig. 3. High-performance liquid chromatogram of (A) nystatin sample 14 and (B) nystatin sample 18.

The majority of the samples of nystatin examined produced a chromatogram similar to that shown (Fig. 4B), though peak 1 was often absent. Based on the measurement of peak areas the major component nystatin $A_1$ (peak 5) comprised 55.0 to 75.0% of the total in most samples. Chromatograms of two of the more complex nystatin samples are shown in Fig. 3); the amount of nystatin $A_1$ found in these materials ranged from 3.5 to 12.0%. The two samples of polyfungin were similar to the nystatin complexes, though the content of nystatin $A_1$ was higher (40.0 and 60.0%); a chromatogram of polyfungin is shown in Fig. 1). For comparison the relative proportions of the main peaks in some of the samples of nystatin and polyfungin are shown in Table I. Spectrophotometric examination indicated that the main components detected in the nystatin complex were tetraenes.

The presence of a heptaene in nystatin has been shown by previous workers[2,3]. We calculated the amount of heptaene in the samples by measuring the absorbance at 382 nm. Using $E_1^{1\%}{}_{cm}$ of amphotericin B[7] as 1670 the content of the heptaene in the samples of nystatin was found to range from 0.5 to 2.0%, the more complex nystatins contained not more than 0.5%. When the eluent was monitored at 382 nm two or three small peaks were detected, the main peak having a retention time of 33.25 min. The presence of these low levels of a heptaene would not affect the chromatograms monitored at 304 nm.

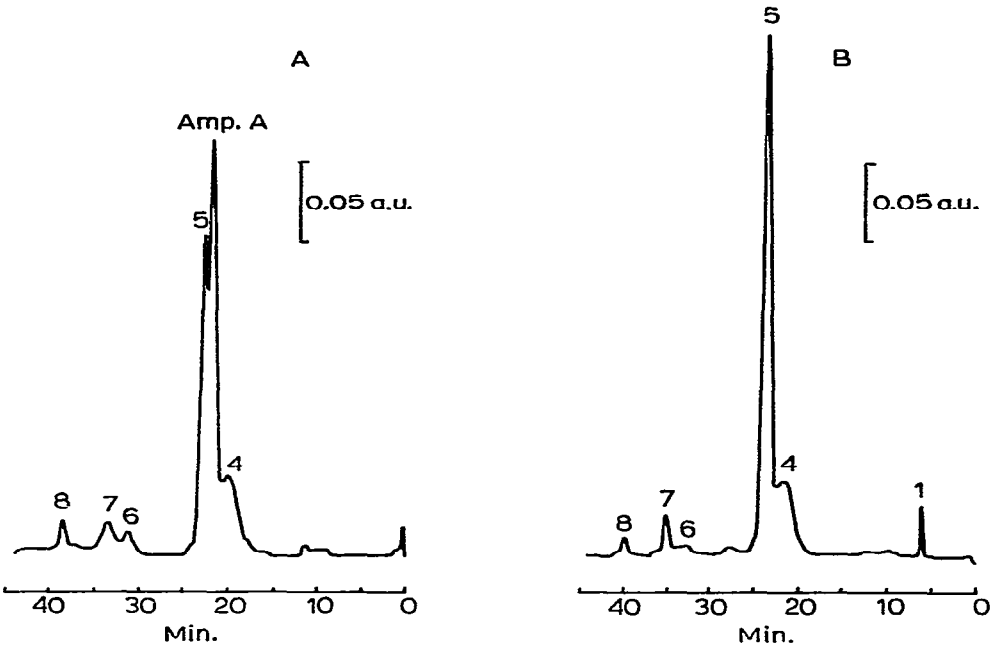


Fig. 4. High-performance liquid chromatogram of (A) mixture of nystatin sample 1 and amphotericin A, and (B) nystatin sample 3.

TABLE I

RELATIVE COMPOSITION OF SAMPLES OF NYSTATIN AND POLYFUNGIN BASED ON PEAK AREAS OF MAIN COMPONENTS SEPARATED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

| *Sample* | | *Peak area of main components (%)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Peak number/retention time (min)* | | | | | | | |
| | | *1/6.83* | *2/12.42* | *3/19.58* | *4/22.00* | *5/24.16* | *6/32.33* | *7/35.50* | *8/39.66* |
| Nystatin | 1 | — | — | — | 19.82 | 71.40 | — | — | 6.28 |
| | 2 | 9.92 | — | — | 18.36 | 56.89 | — | — | 12.12 |
| | 14 | — | 7.32 | 6.21 | — | 11.77 | 7.76 | 19.33 | 33.06 |
| | 15 | — | 5.45 | 4.74 | — | 12.32 | 8.77 | 19.99 | 34.21 |
| | 16 | 7.54 | 2.29 | 2.69 | — | 6.15 | — | 44.09 | 23.69 |
| | 17 | 6.23 | 1.51 | 1.95 | — | 8.12 | — | 49.31 | 21.19 |
| | 18 | 5.13 | 2.63 | 2.67 | — | 6.57 | — | 42.09 | 25.67 |
| | 19 | 2.44 | 1.91 | 2.22 | — | 7.28 | — | 48.58 | 24.61 |
| | 20 | — | 2.66 | 4.20 | — | 3.41 | — | 49.98 | 27.34 |
| Polyfungin | 1 | — | — | — | 12.81 | 40.64 | 12.58 | 14.59 | 5.33 |
| | 2 | — | — | — | 5.45 | 61.42 | 7.36 | 17.09 | 4.37 |

## CONCLUSIONS

Three thin-layer chromatographic methods have been used for the identification of nystatin and to demonstrate heterogeneous composition of some nystatin samples. A high-performance liquid chromatographic method was developed for the qualitative and semi-quantitative evaluation of nystatin. Examination of a number of nystatin samples from different sources revealed that there was a great variation in the composition of pharmaceutical grade material. The more complex nystatins originated from one source. Pharmaceutical grade nystatin is expected to consist largely of nystatin $A_1$ though there is no confirmatory test in the pharmacopoeia[6]. Our results indicate the need for a test to show that pharmaceutical grade material does consist largely of nystatin $A_1$.

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