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Note

Separation of filipin and nystatin complexes by semi-preparative and microbore high-performance liquid chromatography

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It is now widely recognized that antifungal polyene antibiotics are often complex mixtures of closely related compounds[1,2], and high-performance liquid chromatography (HPLC) is the method of choice to analyse and purify them[2,3]. Such a purification becomes more necessary as studies on the mechanism of action of these antibiotics become more precise, in particular, the inducement of permeability in various cell membranes[4,5].

Some of the components of these two antibiotics were isolated by thin-layer or classical chromatography[6,7]. The main component of each antibiotic[8,9] and three filipin impurities were identified[10]. Reversed-phase HPLC separations of filipin[11] and nystatin[4,5,12] have been reported. We report here on the HPLC behaviour of these antibiotics, on both reversed- and normal-phase systems, and on their semi-preparative HPLC purification. Microbore columns were used for the analytical work because of their high efficiency and their low sample and mobile phase consumption.

EXPERIMENTAL

Apparatus

For the analytical work, the chromatograph comprised a Gilson 302 pump, a Rheodyne 7413 sampling valve fitted with a 1-μl internal loop and a Kratos 769 spectrophotometric detector equipped with 5-μl micro flow cells. For the semi-preparative fractionation, the chromatograph comprised a Waters M 510 pump, a Waters U6K injector fitted with a 2-ml loop and a Waters M 481 spectrophotometric detector.

Materials

Filipin was purchased from Sigma (stated purity: *ca.* 40%); nystatin was a gift from Squibb. The impurity contents based on peak area measurements (Figs. 1B and 2A) are 40 and 35% for filipin and nystatin respectively.

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The columns for the analytical work were a 50 cm × 1 mm I.D. microbore packed with Zorbax B.P. Sil, 7–8 μm (DuPont), and a 25 cm × 1 mm I.D. microbore packed with Rosil C_{18} HL, 3 μm (Alltech); both columns were packed as described previously[13]. For the semi-preparative work, a 50 cm × 9 mm I.D. column packed with Partisil ODS 2, 10 μm (Whatman), was employed. The solvents used were distilled water, analytical reagent grade dimethylformamide (DMF) and acetic acid, methanol and dichloromethane for liquid chromatography.

Procedures

Analytical chromatography. In reversed-phase chromatography, methanol–water mixtures from 100:0 to 55:45 (v/v) were tested isocratically as eluents. Experiments were also made by addition of 0–2% acetic acid and 0–10% DMF. The flow-rate was 40 μl/min. In adsorption chromatography of filipin, methanol–dichloromethane mixtures from 95:5 to 85:15 (v/v) were tested isocratically as eluents. The effects of the silica gel activity and of a small addition of acetic acid were also tested. For nystatin, methanol–water–DMF mixtures with 65–100% methanol, 1–10% water and 0–25% DMF were tested. The best results are shown in Figs. 1 and 2; the samples contained 1 mg of antibiotic per ml of eluent. Continuous flow detection was performed at 322 nm for filipin and at 308 nm for nystatin.

Semi-preparative chromatography. The mobile phase composition selected for reversed-phase HPLC was methanol–water–DMF–acetic acid (55.6:34.4:9:1) for filipin and methanol–water–acetic acid (66:33:1) for nystatin. The flow-rate was 8 ml/min. The samples were first dissolved in a small volume of DMF (10%), then the methanol–water mixture was added, giving 20 mg per ml of the eluent. They were filtered through a 1-μm Nuclepore membrane. The volumes injected were usually 200

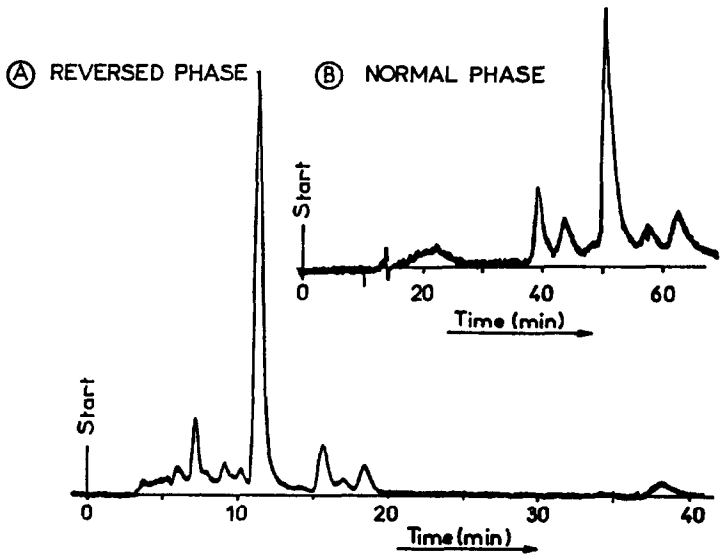

Fig. 1. Microbore HPLC separation of filipin (Sigma). Eluents: (A) methanol–water–DMF–acetic acid (57:37:5:1), flow-rate 40 μl/min; (B) dichloromethane–methanol–water–acetic acid (88.3:11:0.4:0.3), flow-rate 30 μl/min.

μl (4 mg of antibiotic). The effluents were manually fractionated and analysed under conditions given in Figs. 2A and 1B. For nystatin, which is unstable in acidic aqueous solutions[7], the fractions were immediately frozen. Methanol was removed with a vacuum rotary evaporator at 40°C. For nystatin (no DMF in eluent), the fractions were directly lyophilized. For filipin, water was removed by addition of toluene (giving an azeotropic mixture) and evaporation with a vacuum rotary evaporator; the remaining DMF was removed by distillation under vacuum from a trap at −40°C to a trap at −196°C.

RESULTS AND DISCUSSION

Filipin

It is seen from Fig. 1, the filipin complex is comprised of at least ten components. The main component corresponds to the third peak of the normal-phase chro-

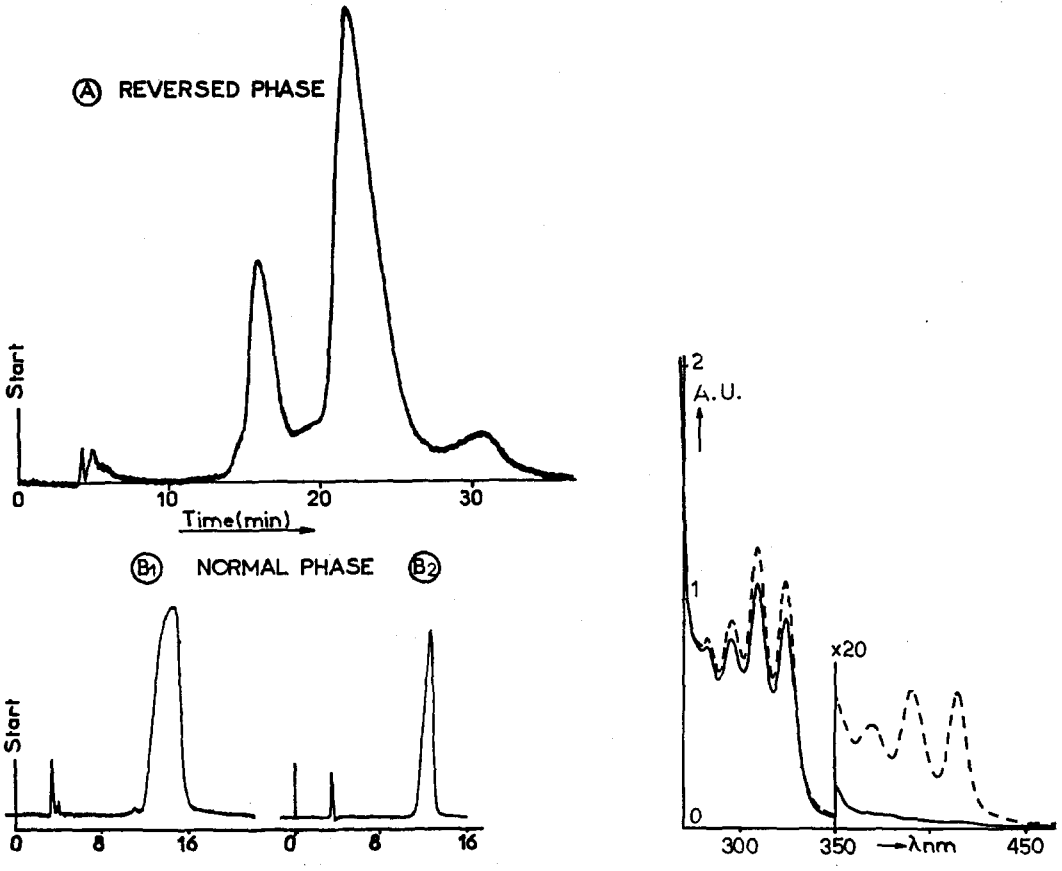

Fig. 2. Microbore HPLC separation of nystatin (Squibb). Eluents: (A) methanol–water–acetic acid (65:34:1), flow-rate 40 μl/min; (B1) methanol–water–acetic acid (95:4.5:0.5), flow-rate 125 μl/min; (B2) methanol–DMF–water–acetic acid (72:25:3:0.4), flow-rate 125 μl/min.

Fig. 3. Ultraviolet absorption spectra of nystatin before (---) and after (——) purification (10^{-5} *M* in DMF).

matogram (Fig. 1B). The reversed-phase chromatogram (Fig. 1A) resembles one reported recently[11], but the quantitative composition is different. The separation efficiency is better with a normal-phase system (Fig. 1B); however, in this case, sample solubility in the eluent is poor and for semi-preparative purposes a reversed-phase system was preferred. About 25 mg of the major component were recovered from 22 injections of 4 mg of filipin (Sigma); the impurities represent no more than 7–8% of all the peak areas.

Nystatin

The reversed-phase separation is shown in Fig. 2A. Two main impurities are detected; furthermore, a slightly retained (polar) component appears. This result is in agreement with previous work[2,4,12]. The non-polar impurities are better detected in normal-phase HPLC (Figs. 2B1 and 2B2), since they are eluted as non-retained peaks. However, the nystatin retention is very strong and the transfer kinetics is slow (owing to the carboxylic and amine moieties). This behaviour is improved by adding DMF in the eluent which increases the sample solubility at the expense of selectivity (Fig. 2B2). As with filipin, it was shown that a small amount of acetic acid improves the peak shape. The reversed-phase conditions were chosen for the semi-preparative purification. About 27 mg of the major component were recovered from 18 injections of 8 mg of nystatin (Squibb); the impurities represent no more than 10% of all the peak areas. An UV absorption spectrum (Fig. 3) shows that the original nystatin contains an heptaene which is eliminated by the HPLC fractionation. Such an impurity had been found in previous work[2,4,12]; using the extinction coefficient of Amphotericin B, the content of this heptaene would be around 1%.

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