ELSEVIER

Journal of Chromatography A, 672 (1994) 247–253

JOURNAL OF CHROMATOGRAPHY A

Short Communication

High-performance liquid chromatographic separations of nystatin and their influence on the antifungal activity ☆

B. Sauer, R. Matusch *

Institut für Pharmazeutische Chemie, Philipps-Universität, Marbacher Weg 6, D-35032 Marburg/Lahn, Germany

(First received October 12th, 1993; revised manuscript received March 22nd, 1994)

Abstract

Several efficient analytical HPLC methods for the separation and comparison of the nystatin complex have been developed. These separations have been extended to the preparative scale in order to gain sufficient quantities of the purified nystatin components for testing antifungal activity. For the first time we describe a new HPLC method which does not affect the antifungal activity of nystatin.

1. Introduction

Polyenic antibiotics are very important substances for the treatment of fungal infections. The first member of this group of antibiotics was nystatin (Fig. 1), which was discovered in 1950 by Hazen and Brown [1]. It is produced by *Streptomyces noursei* and can be separated from the culture broth by extraction with organic solvents [2].

As a result of this methodology commercial available nystatin —as most of the other polyene antibiotics— is a complex mixture of closely related compounds. In order to obtain sufficient quantities of single, well purified antifungal substances it is therefore necessary to develop a method for the separation of the nystatin complex, which is rapid and selective and also preserves the antifungal activity of the isolated compounds.

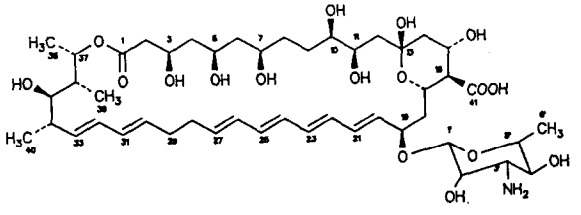

Fig. 1. Structure of nystatin A_1.

During the last twenty years methods using HPLC turned out to be the most efficient and reliable separation techniques for the isolation and analytical comparison of polyene antibiotics. Mechlinsky and Schaffner [3] were the first to publish analytical HPLC methods for the separation of polyene antibiotics. Hansen and Thomsen [4] described a HPLC technique for the comparison of some polyene antibiotics using phosphate buffer–methanol eluents as mobile phase. Thomas *et al.* [5] as well as Margosis and Aszalos [6] used mobile phases with reduced pH values by applying different buffers. Several authors described HPLC methods using acetonitrile instead of methanol [7–10].

* Corresponding author.

☆ Dedicated to Professor Dr. B. Unterhalt, Münster, Germany, on the occasion of his 60th birthday.

0021-9673/94/$07.00
SSDI 0021-9673(94)00291-G

However, none of these analytical systems was adapted to the preparative scale, since the use of buffers causes great difficulties in removing the solvent after the separation, a step which is essential for further investigations with the separated substances. So far there are only two semi-preparative methods, which have been described by Gareil *et al.* [11] and by Milhaud *et al.* [12] who used methanol–water–dimethyl sulphoxide and methanol–water–dimethylformamide–acetic acid as mobile phases, respectively. However, both publications do not give any information about the antifungal activity after the separation.

In this paper we present three different efficient analytical HPLC methods, which are all extended to the preparative scale. Furthermore, we discuss the results of the microbiological assays, which were carried out with the different fractions after the separation. Finally, we present a rapid preparative HPLC method, that allows the purification of high amounts of nystatin and does not affect the antifungal activity of the isolated substances.

2. Experimental

2.1. Instruments

For the analytical separations we used a liquid chromatograph consisting of the following components all supplied by Waters (Milford, MA, USA): HPLC pump type 600 E, photodiode array detectors types 990 and 991 and graphic plotter. Data processing was conducted on a NEC APC III and IV.

The preparative separations were carried out on a liquid chromatograph consisting of two preparative ERC pumps type 64, an ERC UV detector type 7210 (Alteglofsheim, Germany) and a potentiometric recorder produced by Schoeffel (Westwood, NY, USA). Both chromatographs were equipped with a Rheodyne (Cotati, CA, USA) injection valve.

The solvents of the separated fractions were removed using a membrane pump and a vacuum controller constructed by Vaccuubrand (Wertheim, Germany).

2.2. Instrument operation

The detectors were started half an hour before the operation of the chromatograph. The column packings were conditioned with about 15 column volumes of the solvent system. After reaching a constant baseline on the screen or on the recorder, the samples were injected using a Hamilton HPLC syringe (Reno, NV, USA).

2.3. Solvent systems and columns

For the analytical separations we used two columns (250 × 4.0 mm), packed with LiChrospher RP-18 (5 μm) material or with LiChrosorb RP-18 (7 μm) material, respectively, both supplied by Merck (Darmstadt, Germany). The preparative separations were carried out on a column (250 × 25 mm), packed with LiChrosorb RP-18 (7 μm) material and supplied by Merck.

The following mobile phases were used: (I) methanol–water (90:10) + 1% formic acid, pH 3.5; (II) acetonitrile–trifluoroacetic acid 0.02 *M* (50:50), pH 2.7; (III) methanol–water–dimethylformamide (70:20:10), pH 7. All solvent mixtures were degassed with helium before operating the chromatograph.

2.4. Chemicals

The organic solvents were of analytical-reagent or LiChrosolv quality and were supplied by Merck. The water was bidistilled or purified by using a Seralpur Pro 90 C instrument of Seral (Ransbach-Baumbach, Germany). Nystatin was supplied by Serva (article No. 29870; Heidelberg, Germany). The samples were prepared by dissolving nystatin in the solvent mixture to concentrations of 1–5 mg/ml for analytical separations and 10 mg/ml for preparative separations.

2.5. Microbiological assay

Microbiological assays, measuring the activity against *Candida albicans* ATCC 10231 and *Candida tropicalis* ATCC 70156, were conducted by

Heumann (Nürnberg, Germany) using the agar-dilution method DIN 58940.

3. Results and discussion

3.1. Chromatography

The aim of this investigation was to develop a HPLC method which allows the separation of high amounts of nystatin without affecting the antifungal activity of the isolated substances. Therefore we had to take into consideration the poor solubility of the polyene antibiotics in most organic solvents when choosing the mobile phase. On the other hand the mobile phase had to be easily removable since the analytical method had to be extended to the preparative scale. Nystatin, as most of the polyene antibiotics, is an ampholyte (Fig. 1). Therefore its solubility is strongly pH-dependent. Solvent systems with lowered pH values have been shown to be useful for the separation of polyene antibiotics [5,6]. As it was not possible to use a buffer because of the difficulties in removing it after the separation, we chose two organic acids —formic acid (pK_a =

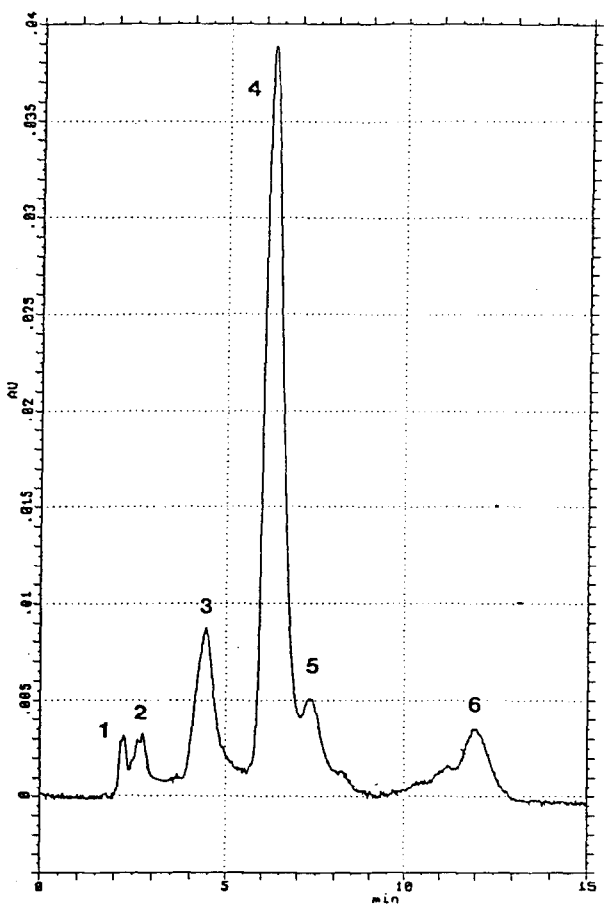

Fig. 2. Analytical chromatogram of nystatin. Stationary phase, LiChrosorb RP-18 (7 μm), 250 × 4.0 mm; mobile phase, methanol–water (90:10) + 1% formic acid, pH 3.5; flow-rate, 1 ml/min; UV at 340 nm; injection volume, 10 μl; sample, 1 mg/ml solution in the solvent mixture. Peaks numbered for comparison with other figures.

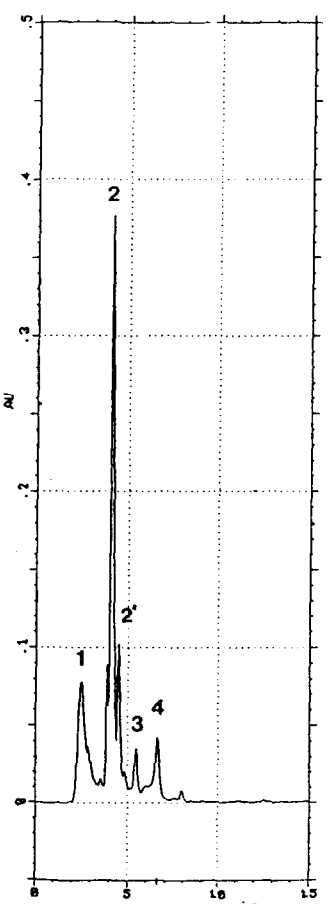

Fig. 3. Analytical chromatogram of nystatin. Stationary phase, LiChrospher RP-18 (5 μm), 250 × 4.0 mm; mobile phase, acetonitrile–trifluoroacetic acid 0.02 *M* (50:50), pH 2.7; flow-rate, 1 ml/min; UV at 340 nm; injection volume, 20 μl; sample, 5 mg/ml solution in the solvent mixture.

3.7) and trifluoroacetic acid ($pK_a = -3$)—which can both easily be removed together with the organic solvent. A major disadvantage of these solvent systems is the instability of polyene antibiotics in solutions with reduced pH values, and it is therefore necessary to work quickly and carefully.

To overcome this problem, we used a third mobile phase with pH 7 consisting of a methanol–water–dimethylformamide mixture, since dimethylformamide is a good organic solvent for polyene antibiotics.

Figs. 2–4a show the analytical chromatograms of nystatin and Fig. 4b its preparative chromatogram under different experimental conditions. All three solvent systems are very useful for the analytical HPLC of nystatin and allowed the separation of five to six peaks in a short time (12–18 min). The chromatogram in Fig. 4a was recorded at two different wavelengths (305 nm, 385 nm) in order to show the deviating structure of peak 5, which is a heptaene, whereas the main peak 3 is a tetraene and corresponds to nystatin A_1. The difference in the polyenic chain of the molecules is expressed in the very characteristic UV–Vis spectra, which are shown in Fig. 5. The

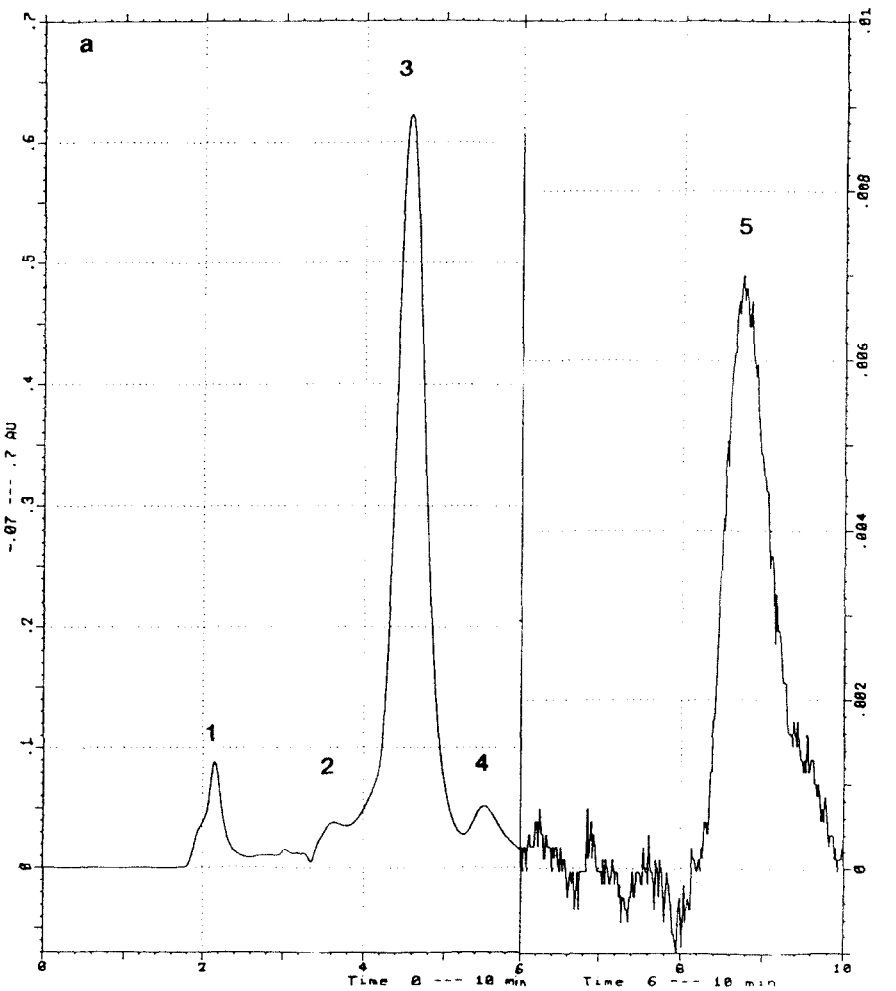

Fig. 4.

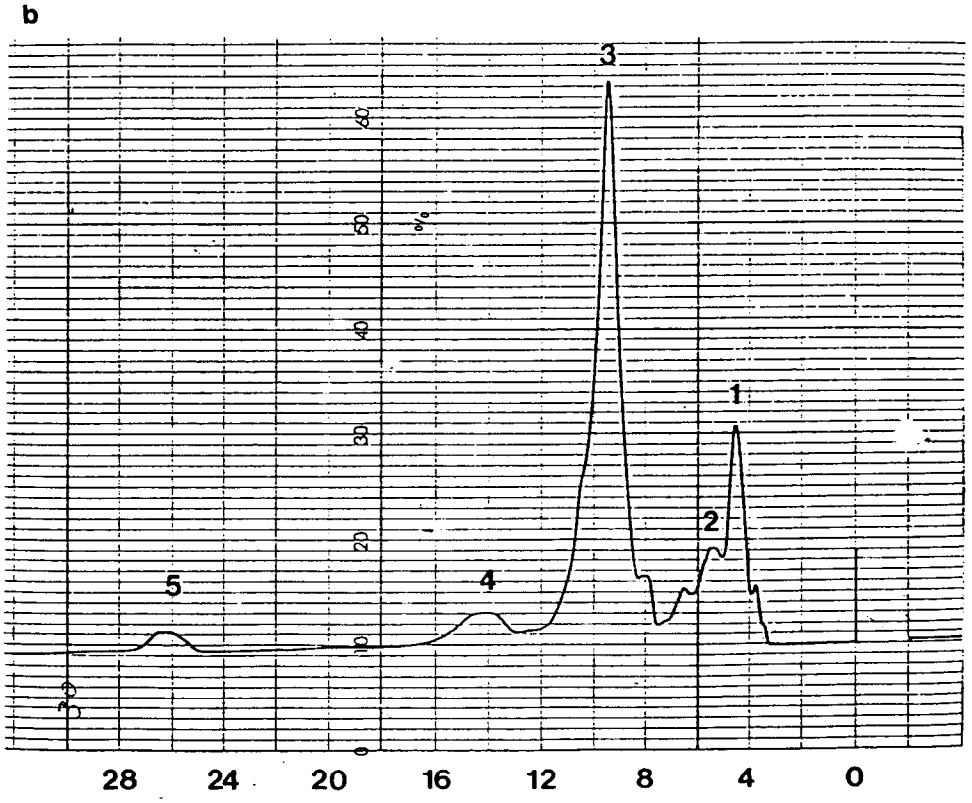

Fig. 4 (a) Analytical chromatogram of nystatin. Stationary phase, LiChrosorb RP-18 (7 μm), 250 × 4.0 mm; mobile phase, methanol–water–dimethylformamide (70:20:10); flow-rate, 1 ml/min; UV at 305 (left) and 385 (right) nm; injection volume, 5 μl; sample 1 mg/ml solution in the solvent mixture. (b) Preparative chromatogram of nystatin. Stationary phase, LiChrosorb RP-18 (7 μm), 250 × 25 mm; mobile phase, methanol–water–dimethylformamide (70:20:10); flow-rate, 15 ml/min; UV at 340 nm; injection volume, 5 ml; sample, 10 mg/ml solution in the solvent mixture.

presence of a heptaene in some nystatin complexes has been observed before [3,13] but its structure is still unknown.

The preparative chromatogram also shows a separation of five fractions in 28 min (Fig. 4b). Although there is an overlapping of some fractions, at least the main fraction (nystatin A_1) and the last fraction (the heptaene component) can be purely separated. This was checked by NMR spectroscopy and analytical HPLC with diode array detection. This method is also very efficient, as high amounts of nystatin can be purified by one separation (50 mg).

3.2. Microbiological assay

After the separation of 100 mg of nystatin with the different methods microbiological assays were carried out on the collected fractions in each case. Table 1 shows the minimal inhibitoric concentrations of the tested fractions against *Candida albicans* and *Candida tropicalis*. It is evident that the purification with the methanol–water–dimethylformamide mixture (pH 7) does not affect the antifungal activity of nystatin A_1 at all, whereas after the separation with the two acidic solvent systems all fractions have lost their antifungal activity (MIC of the reference = 2 μg/ml). Obviously the low pH value causes an

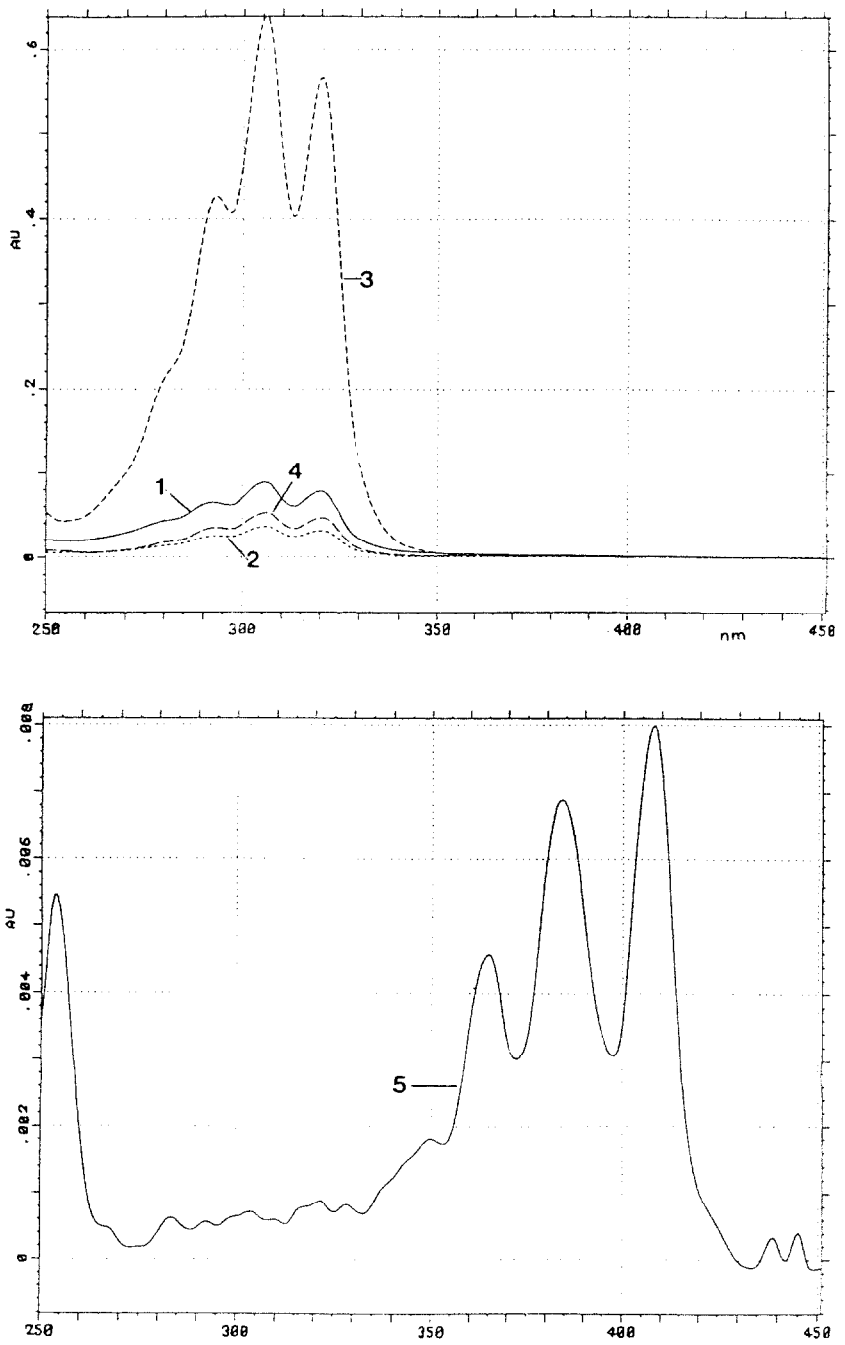

Fig. 5. UV–Vis spectra of the peaks 1–5 shown in Fig. 4a.

Table 1
Minimal inhibitory concentrations (MIC) of the nystatin fractions shown in Fig. 4b

Fraction	MIC (g/ml)	
	Candida albicans	*Candida tropicalis*
Fraction 1/2	>125	>125
Fraction 3	2	4
Fraction 4	3	4
Fraction 5	8	8
Reference (nystatin)	2	2

alteration in the structure of the molecules so that they are no longer able to form complexes with the membrane sterols, which is discussed to be their mode of action [14].

4. Conclusions

In conclusion, our data show that although all three presented HPLC methods are rapid and efficient for an analytical separation of nystatin, only the one using a methanol–water–dimethylformamide mixture with neutral pH value is applicable to the preparative purification of nystatin, since it is the only system which does not affect the antifungal activity of the separated components. The use of this method allows the separation of the nystatin complex into five fractions, of which the main component nystatin A_1 (fraction 3) and the heptaene component (fraction 5) can be obtained purely.

References

[1] E.L. Hazen and R. Brown, *Science*, 112 (1950) 423.
[2] G.W. Michel, in K. Florey (Editor), *Analytical Profiles of Drug Substances*, Academic Press, New York, 1977, Vol. 6, p. 341.
[3] W. Mechlinsky and C.P. Schaffner, *J. Chromatogr.*, 99 (1974) 619.
[4] S.H. Hansen and M. Thomsen, *J. Chromatogr.*, 123 (1976) 205.
[5] A.H. Thomas, P. Newland and G.J. Quinlan, *J. Chromatogr.*, 216 (1981) 367.
[6] M. Margosis and A. Aszalos, *J. Pharm. Sci.*, 73 (1984) 835.
[7] P. Helboe, M. Thomsen and S.H. Hansen, *J. Chromatogr.*, 189 (1980) 249.
[8] J.W. Mayhew, C. Fiore, T. Muray and M. Barza, *J. Chromatogr.*, 274 (1983) 271.
[9] A.H. Thomas and P. Newland, *J. Chromatogr.*, 354 (1986) 317.
[10] M.B. Swami, M.K. Sastry, A.G. Nirgudkar and R.K. Nanda, *Hind. Antibiot. Bull.*, 25 (1983) 81.
[11] P. Gareil, G. Salinier, M. Caude and R. Rosset, *J. Chromatogr.*, 208 (1981) 365.
[12] J. Milhaud, P. Gareil and R. Rosset, *J. Chromatogr.*, 358 (1986) 284.
[13] N. Porowska, L. Malski, Z. Plociennik, D. Kotiuszko, H. Morawska, Z. Kowszyk-Gindifer and H. Bojarska-Dahling, *Adv. Antimicrob. Antineoplast. Chemother.*, 1 (1972) 1031.
[14] R.W. Holz, *Ann. N.Y. Acad. Sci.*, 235 (1974) 469.