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SEPARATION OF POLYENE ANTIFUNGAL ANTIBIOTICS BY HIGH-SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

High-speed liquid chromatography was applied to separate the components of different classes of polyene macrolides. The instrument contained a UV detector and VYDAC RP packing and a solvent mixture of water–methanol–tetrahydrofuran (420:90:45–90, v/v) was employed. Optimum conditions for the separation of different antibiotics were achieved by changing the amount of tetrahydrofuran in the solvent mixture. Eurocidin separated completely in 5 min into two components, which were quantitated from peak height measurements. In less than 20 min, candicidin separated into five components with better resolution than that obtained after 600 transfers with counter-current distribution separation. The reproducibility of retention times was very good. It was observed that antibiotics with longer retention times, as measured under identical conditions, were also more active as antifungal agents. The heptaenes appeared to form three distinct groups with characteristic similar ranges of retention time and levels of antifungal activity.

INTRODUCTION

The polyene macrolide antifungal antibiotics[1,2] are produced by over 10% of all antibiotic-producing Streptomyces species and were introduced a quarter of a century ago with the discovery of nystatin. Since then, over sixty members have been added to this group of microbial products. They are classified as tetraenes, pentaenes, hexaenes or heptaenes according to the number of conjugated double bonds in their chromophore and its characteristic absorption spectrum in the ultraviolet–visible region.

These antibiotics are very active against fungal infections and have found use in medicine and agriculture. In addition to their antifungal activity, some antibiotics also exhibit antiprotozoal activity and have a significant effect on steroid metabolism[3] in man and animals. The polyene antibiotics show little, if any, activity against bacteria, and this property sets them apart from the better and longer known antibacterial macrolide antibiotics[2] to which they are chemically related.

Because of their very characteristic light absorption, the polyene macrolides were relatively easy to discover and divide into the appropriate chromophore groups. Within each group, however, the differentiation of members was significantly more

complicated and strongly dependent on the use of paper chromatography (PC), thin-layer chromatography (TLC)[4], column chromatography[4], counter-current distribution (CCD)[5] and also gas chromatography[6] and chemical degradation.

The results of these procedures were, at best, often only partially successful and left a significant number of these antibiotics unresolved as to their exact identity and homogeneity. These antibiotics were usually the most complicated chemically and exhibited the highest molecular weights. This ambiguity in identity is especially acute with the chemically poorly defined and biologically important heptaene macrolide antibiotics[5].

It has therefore been realized for some time that further progress in the field of isolation, identification and chemical elucidation of the more complex members of the polyene macrolide antibiotics, such as candicidin and trichomycin, would require a more efficient, rapid and reliable separation technique.

Past experience from studies involving liquid–liquid partition chromatography and counter-current distribution indicated that a substantial increase in the number of theoretical plates coupled with a shorter separation time, as is characteristic for high-speed liquid chromatography (HSLC), could be the answer to the problem. This latter technique has been applied successfully in the separation of many complex mixtures of natural products, including recently the separation of some antibacterial macrolide antibiotics[7].

In a previous report[8], we described the separation of some heptaene macrolides by HSLC. This paper describes in detail the method and its application to all chromophore classes of the polyene macrolide antibiotics.

EXPERIMENTAL

Instruments

A non-commercial liquid chromatograph was constructed from components supplied by Waters Ass. (Framingham, Mass., U.S.A.) and Laboratory Data Control (LDC) (Riviera Beach, Fla., U.S.A.). The LDC pumping system of the chromatograph consisted of a Milton Roy 3000 p.s.i. maximum reciprocating pump equipped with a pulse dampener and was connected in sequence to a septum injector, chromatographic column, UV detector and a waste reservoir. All connections were made by means of stainless-steel Swagelok fittings and 1/16-in. O.D. × 0.009-in. I.D. stainless-steel tubing. The septum injector (Waters Ass.) contained one EPR septum disc. Chromatographic columns (Waters Ass.) were made of stainless-steel tubing (3 ft. × 1/8 in. O.D. × 0.093 in. I.D.) and were packed by standard methods. The column was maintained thermostatically at $+30^\circ$ by means of a water-jacket connected to a constant-temperature Haake circulator, Model ED. The UV detector was an LDC phosphor converted to a 350-nm UV monitor featuring an 8-μl cell and double-beam optical system. The measurements were recorded by a flush-mounted 10-in chart Servo/Riter II potentiometric recorder produced by Texas Instruments (Houston, Texas, U.S.A.).

Instrument operation

The UV detector and the Haake circulator were started 1 h before the operation of the chromatograph. After this time, the pumping system was started and set at a flow-rate of 0.9 ml/min, as measured by a 10-ml burette in 10-min time intervals. The

pressure in the pumping system was 1000–1200 p.s.i., depending on the solvent composition. The attenuation of the UV monitor was usually kept at 0.04 or 0.02 unit full-scale (Aufs), and the chart speed of the recorder was constant at 15 in./h. After achieving equilibrium in the instrument, demonstrated by a constant baseline on the recorder, a sample, usually 1–5 μl containing 0.1–10 μg of an antibiotic, was injected on-stream through the injector using a 10-μl Precision Sampling (Baton Rouge, La., U.S.A.) syringe. In order to achieve good separation, it was necessary to wash new column packings with a large volume of an appropriate solvent system under operation conditions.

Solvent systems and column packings

Methanol, acetonitrile and tetrahydrofuran (THF) of certified A.C.S. grade (Fisher Scientific, Pittsburgh, Pa., U.S.A.) were mixed with distilled water in different proportions to produce three solvent systems useful in the separation of the different polyene antibiotics under study on octadecyl permanently coated packing materials:

I: water–methanol–THF (420:90:45–90, v/v);

II: water–methanol–acetonitrile (200:200:80–100, v/v);

III: water–methanol (10–50% water, v/v).

Two commercially available packings were employed: VYDAC RP, 30–44 μm, and Bondapak C_{18}/Corasil I, 37–50 μm (Waters Ass.). Between these packings, differences in absorption power were observed, and solvent adjustments had to be made accordingly to produce separation. On VYDAC RP packing, the best separation was achieved with solvent system I. The proportion of THF in the mixture was changed between 45 and 90 parts, depending on the properties of the antibiotic to be examined. For the same antibiotic, an increase in the amount of tetrahydrofuran decreased the retention time, and *vice versa*. On the Bondapak C_{18}/Corasil I packing, which showed stronger antibiotic absorption, solvent systems II and III were more suitable. However, the resolution obtained was generally poorer than on the other packing and all results presented here were made utilizing the VYDAC RP material and solvent system I. In addition to the above packings, Bondapak Phenyl/Corasil, 37–50 μm (Waters Ass.), was examined with all three solvent systems and a variety of polyene antibiotics, but this packing failed to give good separations. Prior to use, all solvent systems were de-gassed by a vacuum technique.

Antibiotics

The antibiotics included in this study and their sources are listed in Table I.

Standards of the antibiotics for HSLC use were prepared by dissolving 10 mg of each in 2 ml of dimethyl sulfoxide and diluting the solution with 18 ml of methanol to a final concentration of 0.5 μg/μl. Higher dilutions were made by adding more methanol. In the case of nystatin, a final concentration of 1 μg/μl was employed and was obtained by dissolving 20 mg of solid by the above procedure.

RESULTS AND DISCUSSION

The polyene antifungal antibiotics have in common many properties which hamper their isolation, characterization and structural determination. The most important are poor solubility in water and organic solvents, extreme sensitivity to

TABLE I

POLYENE MACROLIDE ANTIBIOTICS AND DERIVATIVES TESTED BY HSLC

Antibiotic or derivative	*Chromophore class*	*Source*
Nystatin	Tetraene	E. R. Squibb, New Brunswick, N.J., U.S.A.
Amphotericin B	Heptaene	E. R. Squibb
Filipin	Methylpentaene	Upjohn, Kalamazoo, Mich., U.S.A.
Eurocidin	Pentaene	Takeda Chemical Industries, Osaka, Japan
Trichomycin	Heptaene	Fujisawa Pharmaceutical Co., Osaka, Japan
Hamycin	Heptaene	Hindustan Antibiotics, Pimpri, Poona, India
Mediocidin	Hexaene	Waksman Institute of Microbiology, Rutgers University, New Brunswick, N.J., U.S.A.
Candidin	Heptaene	Waksman Institute of Microbiology
Fungimycin	Heptaene	Waksman Institute of Microbiology
Candicidin	Heptaene	Waksman Institute of Microbiology
N,N'-Diacetylcandicidin	Heptaene	Waksman Institute of Microbiology
N-Acetylamphotericin B	Heptaene	Waksman Institute of Microbiology

heat and pH outside the 6–9 range as well as sensitivity to visible and ultraviolet light and air oxidation.

The HSLC of polyene macrolide antibiotics seemed to offer an ideal analytical method for its speed, sensitivity, reproducibility and non-destructive character. Detection after chromatographic separation presented no problem as these compounds exhibit strong absorption in the ultraviolet–visible region. Table II[9] shows average values of the major absorption bands of the polyene macrolide antibiotics which are characteristic for the different chromophore classes.

TABLE II

AVERAGE VALUES OF MAJOR ABSORPTION BANDS OF THE POLYENE MACROLIDE ANTIBIOTICS[9]

Number of double bonds	*Chromophore class*	*First three $\lambda_{max.}$ (nm)*
4	Tetraenes	291; 304; 318; ±2
5	Pentaenes	317; 331; 350; ±2
5	Methylpentaenes	323; 340; 357; ±2
5	Carbonylpentaenes	364; broad peak
6	Hexaenes	340; 358; 380; ±2
6	Carbonylhexaenes	385; broad peak
7	Heptaenes	361; 382; 405; ±2

In addition to differences in position of absorption bands shown in Table II, the polyene macrolides exhibit differences in molar extinction expressed, conveniently as $E^{1\,\%}_{1\,cm}$. The $E^{1\,\%}_{1\,cm}$ ranges from 700 to 1700 and the response of a UV detector used in HSLC is determined by its sensitivity and the absorption characteristic and concentration of the antibiotic. The UV monitor used in this study was able to measure all chromophore classes of the polyene antibiotics, with maximum sensitivity at 350 nm and lower sensitivity on both sides of this value.

Solvent and column packing selection was based on considerations of the solubility of polyene antibiotics. The compounds are not soluble in non-polar or weakly polar solvents and a simple absorption mode of separation was not available. Partition, on the other hand, is known to produce good results in CCD, TLC and PC and seemed to be feasible also in HSLC.

Although many solvent mixtures and packings could be considered for this purpose, it was decided to employ packings with permanently bonded liquid phases for their simplicity of operation and reliability. The Bondapak C_{18}/Corasil I and the similar material VYDAC RP were the first choices because of the hydrophobic nature of their octadecyl coating, which was expected to interact with the known hydrophobic regions of the polyene macrolides[10]. This type of packing works best in a reverse-phase mode in which the mobile phase is polar, consisting of water usually mixed with methanol, acetonitrile or THF in ratios determined by the properties of the compounds to be separated.

The polyene antifungal antibiotics are known to be soluble in mixtures of water and polar organic solvents such as methanol, acetone, acetonitrile and THF. The solvent system successful in separating these antibiotics by HSLC was found to be water–methanol–THF (420:90:45–90, v/v) when used with the VYDAC RP packing. The proportion of THF in the mixture was very critical, and its increase from 45 to 90 parts significantly increased the solubility of the antibiotics, thereby decreasing the retention time during HSLC separation. Lower proportions of THF in the solvent mixture were used for separating the more soluble antibiotics, including tetraenes, methylpentaenes, pentaenes and N-acetyl derivatives of all polyene macrolide antibiotics. The generally less soluble heptaenes required a higher proportion of THF. The exact amount of THF, however, should be established experimentally in each case, because of differences observed between packings and effects of less defined factors dealing with saturation, temperature, etc.

The Waters Ass. packing, Bondapak C_{18}/Corasil I, showed under similar conditions much stronger retention than the VYDAC RP packing and required a solvent mixture containing a significantly higher proportion of THF or methanol in order to produce elution and separation. It was found practical with this packing to employ different solvent mixtures with a higher proportion of organic solvents which exhibited a better solubility of the antibiotics (solvent systems II and III). As before, the proportion of organic solvents in the mixture affected the solubility and retention time of the antibiotics and was adjusted in the indicated range when necessary. The separations produced on Bondapak C_{18}/Corasil I, however, were inferior to those obtained on the VYDAC RP packing.

Examples of the HSLC separation of different chromophore classes of polyene macrolides obtained with VYDAC RP packing and solvent system I are given below.

Tetraenes

This group and the heptaenes are the most abundant among known macrolide antifungal antibiotics. The tetraenes are sub-divided into small and large macrolactone ring antibiotics[1] and many of their physical properties seem to follow this division. The present study was focused on the large macrolactone ring antibiotics and is represented by nystatin, the first polyene macrolide to be discovered in 1950 and also the most commercially important.

For many years since its discovery, nystatin was recognized as a single, homogenous compound, and a large number of chemical and biological studies with this mixture were made. In 1968[11], however, nystatin was found to contain as much as 30% of a minor component, which caused some concern regarding the data previously reported. Similar problems exist throughout the whole polyene macrolide group.

The separation of nystatin was achieved in 12–14 h by employing the CCD method (200 transfers) and utilizing a solvent system buffered at pH 5.0. This rather long procedure was damaging to the antibiotic because of the time and the low pH involved. The use of HSLC represents a substantial improvement. A separation comparable with the results obtained by CCD but achieved at neutral pH and in less than 15 min is shown in Fig. 1b, whereas Fig. 1a shows an HSLC separation of nystatin at lower resolution. The main component of nystatin designated in the separation curve as component 1 is followed by component 2, both of which are tetraenes. In addition, component 3 was separated from the mixture of nystatin and after spectroscopic examination proved to be a heptaene, the nature of which is obscure. The amount of this heptaene may vary in different batches of nystatin and can be readily measured by UV analysis of the whole mixture. However, no simple procedure for separating the heptaene from the tetraene fraction was previously available.

A quantitative analysis of tetraenes in the presence of heptaenes by HSLC requires some adjustment of the detector sensitivity. As evident from Table II, the absorption maxima of tetraene antibiotics are removed from the peak sensitivity of the UV monitor employed by about 30 nm, whereas the heptaenes are removed by

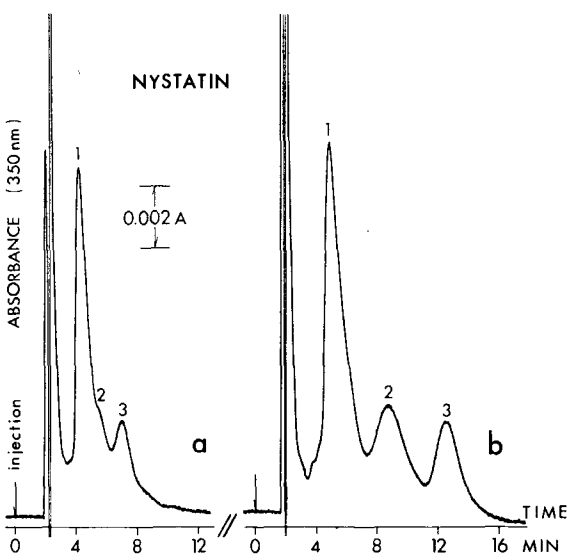

Fig. 1. Separation of nystatin, a tetraene antifungal antibiotic, by HSLC on VYDAC RP column packing and the effect of mobile phase composition on the resolution of components. Tetraene components of nystatin, 1 and 2; heptaene component, 3. Mobile phase: water–methanol–THF in proportions (a) 420:90:60; (b) 420:90:50. Sample size injected: (a) 4 μg/4 μl; (b) 8 μg/8 μl.

only about 10 nm. At similar concentrations, this causes a much weaker response of the detector to tetraenes as to heptaenes and, for the same reason, to all other chromophore classes of the polyene antibiotics. In addition, the tetraenes usually exhibit lower molar extinctions, which will decrease further the detector response. These differences in sensitivity of the UV detector can be readily corrected if needed by utilizing a proper conversion kit and changing the spectral range or perhaps better by using a recently available variable wavelength detector.

The HSLC method of separating nystatin developed here was also applied successfully to another large macrolactone ring tetraene antibiotic, amphotericin A, and a less known tetraene antibiotic, PA-166.

Pentaenes

There are three sub-groups in this class of antibiotics, characterized by the different absorption spectra indicated in Table II. The "typical" pentaenes are the least characterized, and only one antibiotic, eurocidin, was investigated in more detail and its partial chemical structure proposed[12]. From this study, based only on mass spectrometric analysis of degradation products, it became evident that eurocidin consists of two components, A and B. There is no report in the literature on the chromatographic separation of these components.

As shown in Fig. 2a, eurocidin was separated completely into its components by HSLC in about 5 min. The effect of a decreased proportion of THF in the solvent mixture I on the retention time and resolution of eurocidin is demonstrated in Fig. 2b. The sensitivity of the UV detector to eurocidin and, for that matter, to all "typical" pentaenes is shown in Fig. 2c, where as little as 25 ng was detected. Fig. 2c,

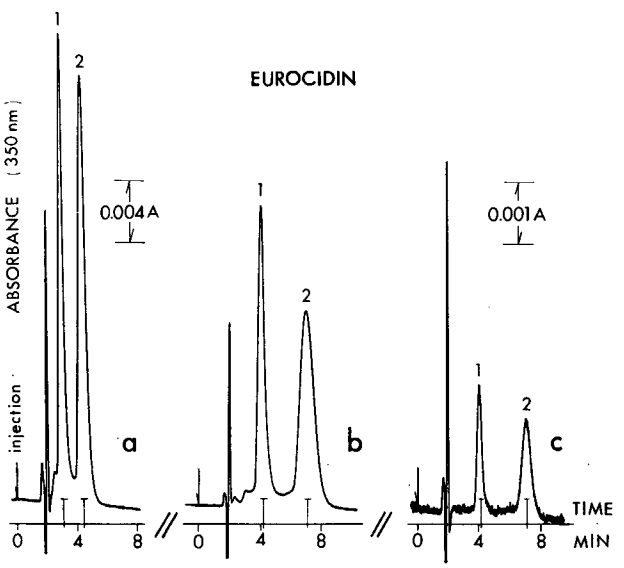

Fig. 2. Separation of eurocidin, a pentaene antifungal antibiotic, by HSLC on VYDAC RP column packing and the effect of mobile phase composition on retention time and resolution. Mobile phase: water–methanol–THF in proportions (a) 420:90:70; (b) and (c) 420:90:60. Eurocidin components, 1 and 2. Sample size injected: (a) and (b) 0.375 μg/3 μl; (c) 0.025 μg/μl.

when compared with Fig. 2b, also illustrates the good reproducibility of retention time. Varying concentrations of antibiotic within the range shown had no effect on the retention time.

Because of the good resolution of eurocidin components by HSLC and the constant retention time obtained, an attempt was made to use the technique developed here for the quantitation of the components. It was found that by measuring only peak heights of the individual components, a reasonable agreement with the known amount of antibiotic was achieved. The corresponding standard curve is presented in Fig. 3.

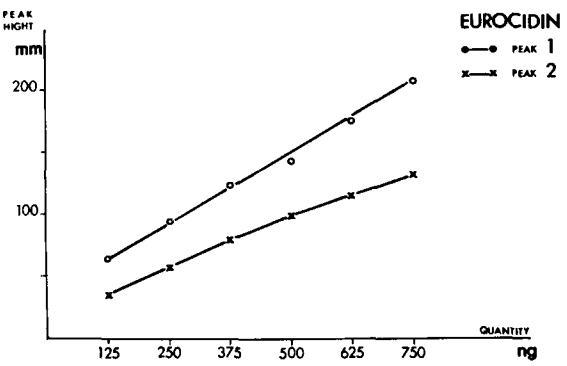

Fig. 3. Relationship between the amount of eurocidin used for HSLC separation and peak heights of components 1 and 2 measured on the separation curve. Conditions of chromatography as shown in Fig. 2b at 0.04 Aufs.

Methylpentaenes

The methylpentaenes were represented in this study by filipin, the best known member of this class. The antibiotic was first described in 1955 and isolated in a "crystalline" form. There are only very few polyene antibiotics pure enough to permit crystallization, and this may have been the reason that for a long time filipin was considered to be a well defined, single compound. As in the case of nystatin and many others, involved chemical and biological studies followed, only to be seriously questioned in 1968 when Bergy and Eble[4] separated filipin into at least eight pentaene components by simple TLC. It was of interest to compare these results with an HSLC separation of filipin. As shown in Fig. 4b, filipin separated into two main components, 1 and 2, in addition to at least three minor components. The same batch of antibiotic tested by TLC by the Bergy and Eble method resolved into three zones, two main and one minor, indicating some similarity to the results obtained with HSLC.

The constant retention time and the decrease of minor component resolution with increased sample size are demonstrated in Fig. 4. Better resolution of larger samples of filipin, which was necessary to analyse the minor components, was obtained after increasing the retention time through solvent system modification. Similarly to eurocidin, quantitative peak height measurements of the two main filipin components separated by HSLC proved to give good agreement with the amount of sample applied.

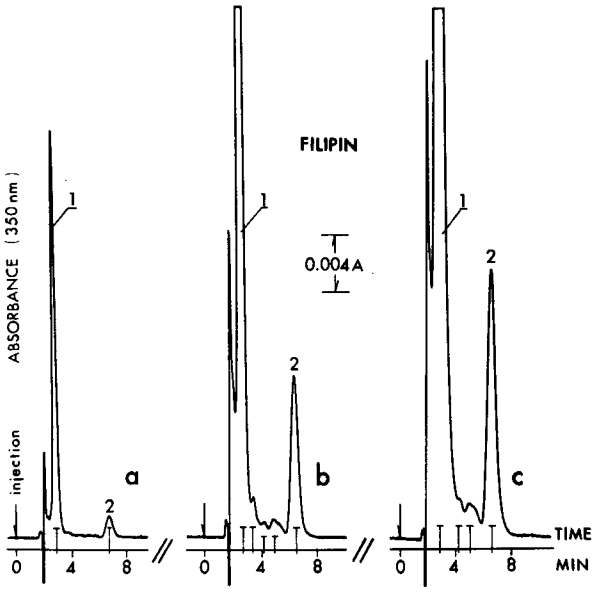

Fig. 4. Separation of filipin, a methylpentaene antifungal antibiotic, by HSLC on VYDAC RP column packing and the effect of sample size on the resolution. The major components of filipin are 1 and 2. The detected major and minor filipin components are marked T. Mobile phase: water–methanol–THF (420:90:60). Sample size injected: (a) 0.125 μg/μl; (b) 1 μg/2 μl; (c) 1.5 μg/3 μl.

Carbonylpentaenes, hexaenes and carbonylhexaenes

The relatively new groups of carbonylpentaenes and carbonylhexaenes[9] were not investigated in this study. The hexaenes, which are poorly defined chemically and are rare among the polyene macrolides, were represented by mediocidin. It was included here mainly to test the response of the UV detector to the characteristic absorption bands of this chromophore group. Separation conditions were not optimized. An HSLC separation curve for this antibiotic is included in Fig. 7.

Heptaenes

This is the largest and most ill-defined polyene macrolide antibiotic group in terms of multiplicity of components and complexity of chemical structure. This group of antibiotics is also the most valuable biologically and difficult to separate. Only limited separations were achieved by TLC and PC. The CCD technique proved to be the most useful for the purification and characterization of these antibiotics.

However, limitations of the CCD method affected progress in the isolation, identification and proper classification of these antibiotics to a great extent, resulting in considerable confusion over the identity of many members. Only a general classification based on chemical degradation seemed to be well established and indicates the presence of non-aromatic and aromatic sub-groups.

The non-aromatic sub-group, represented in this study by amphotericin B and candidin, has fewer members than the aromatic sub-group. The aromatic sub-group is not uniform and can be divided further into heptaenes containing in their molecule a side-chain of *p*-aminoacetophenone or N-methyl-*p*-aminoacetophenone. Other

aromatic heptaenes such as fungimycin form a separate sub-group. The picture of the diversity of heptaenes would not be complete without noting that most of them are mixtures of many components, often belonging to different sub-groups. The number of the components appears to be proportional to the resolution power of the separation methods applied. It was hoped that the application of the HSLC technique would increase our understanding and further development of these antibiotics.

The separation of candidin by HSLC is shown in Fig. 5, and indicates the presence of at least five components. Until now, only three were reported in the literature[13] and were found only after extensive CCD separation. As illustrated in Fig. 5, the resolution of candidin components by HSLC was readily adjusted by changing the proportion of THF in solvent system I.

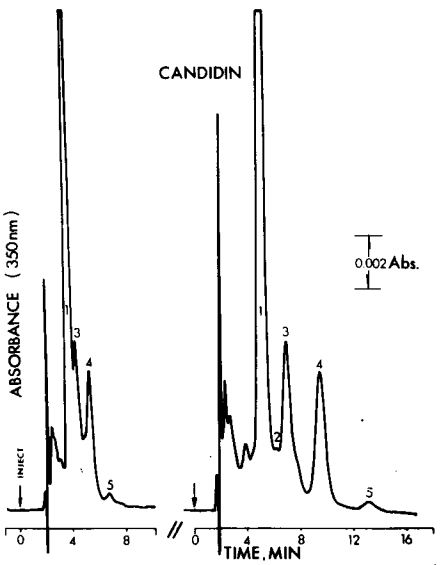

Fig. 5. Separation of candidin, a heptaene antifungal antibiotic, by HSLC on VYDAC RP column packing and the effect of mobile phase composition on the resolution of components. Candidin components are indicated by numbers 1–5. Mobile phase: water–methanol–THF in proportions 420:90:70 (left) and 420:90:60 (right).

Amphotericin B is the most clinically important and best chemically[10] defined of the non-aromatic heptaene antibiotics. This was due in part to the unique purity and homogeneity of amphotericin B. As shown in Fig. 6 with HSLC amphotericin B consists mainly of one component with trace amounts of two other components.

Among the aromatic heptaenes, the following antibiotics are better known: candicidin, trichomycin, hamycin and fungimycin. All were previously subjected to chromatographic analysis, including PC, TLC and CCD, and were found to consist of more than one component. Fungimycin, being a base, was easily distinguished by TLC from the other amphoteric compounds. However, the relationship between trichomycin, hamycin and candicidin was always confusing[5,14].

To compare these antibiotics by HSLC, the separation was conducted under

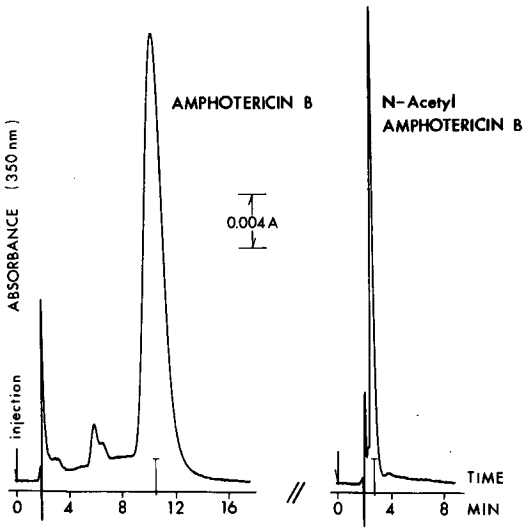

Fig. 6. Separation of amphotericin B, a heptaene antifungal antibiotic, and its N-acetyl derivative by HSLC on VYDAC RP column packing under identical chromatographic conditions. Mobile phase: water–methanol–THF (420:90:45). Sample size injected: amphotericin B, 0.5 μg/μl; N-acetylamphotericin B, 0.0125 μg/μl.

identical chromatographic conditions, allowing a reliable comparison of the corresponding retention times. The separation curves obtained are depicted in Fig. 7 and reveal that the components of trichomycin and hamycin are clearly different from those of candicidin. A closer examination of trichomycin and hamycin, supported by additional HSLC studies, also indicated that these antibiotics are different. A similarity or even identity of the minor components, however, could not be ruled out and will be further investigated.

The separation of candicidin by HSLC into five components, as illustrated in Fig. 7, is noteworthy as very extensive CCD separation studies involving 600 transfers were not able to produce a similar resolution[5]. The presence of numerous components in candicidin was concluded indirectly in the CCD studies from considering the deviation of the experimental separation curve from the theoretical curve for a pure solute.

Polyene macrolide HSLC behavior and antifungal activity

To evaluate the effect of some chemical modifications of the polyene macrolides on their HSLC behavior, the N-acetyl derivatives of amphotericin B and candicidin were examined. It was found that under identical HSLC conditions, the derivatives exhibited a significantly lower retention time than that observed for the parent compounds. This is illustrated in Fig. 6 for N-acetylamphotericin B and in Fig. 7 for N,N′-diacetylcandicidin. Similar studies with the methyl ester derivatives of both antibiotics indicated an increase in the appropriate retention times.

This observation pointed to a possible relationship between HSLC retention time and the antifungal properties of the derivatives examined. The N-acetyl derivatives of polyene antibiotics are known to have lost a considerable part of the antifungal properties of the parent compounds, whereas the methyl ester derivatives have re-

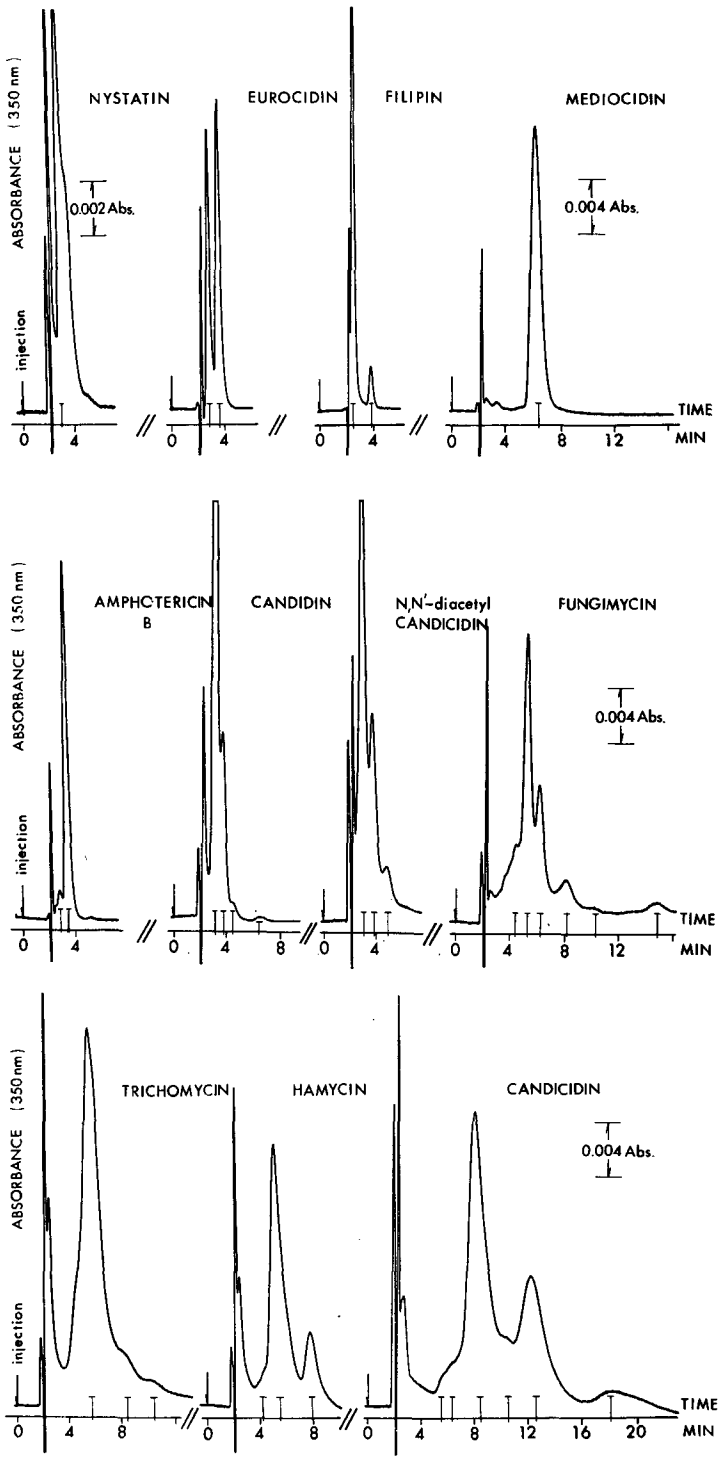

Fig. 7. HSLC separation on VYDAC RP column packing of polyene antifungal antibiotics belonging to different chromophore classes and the comparison of the separation curves obtained under identical conditions. Presence of components in the separation curves is marked T. Mobile phase: water–methanol–THF (420:90:75). Attenuation of the UV detector: 0.04 Aufs or as indicated.

tained full activity[15]. Because the antifungal activity of polyene macrolides is related to their ability to bind with membrane sterol, which is mainly ergosterol in fungal cells and which is strongly hydrophobic, it was reasonable to assume that binding by the hydrophobic coating of the column packing may reflect these properties. Weaker absorption by the column packing and the resultant related shorter retention time by HSLC would characterize lower antifungal activity, and *vice versa.*

With all the limitations of such a simple model in mind, it appeared important to compare also different antibiotics in terms of their retention times and known antifungal activities. The retention time measurements for the antibiotics investigated were made under constant conditions. The corresponding separation curves are shown in Fig. 7. The antifungal activities of these antibiotics were obtained from the literature[9] and are only approximate as many factors, such as antibiotic purity, method of assay and the microorganism itself, may affect the activity reported. Nevertheless, the comparison presented in Table III shows a general agreement between the HSLC retention time and the corresponding antifungal activity of the antibiotics, indicating an increase of activity with increase in retention time. The large deviation of hamycin from this agreement may reflect differences in the methods of bioassay and the purity of the antibiotic studied.

The main components of the heptaene antibiotics in Table III appear to form three distinct sub-groups, characterized by a different range of retention time and antifungal activity. This classification is presented for clarity in Table IV.

Hamycin was included in group B because in this and many other chemical tests it resembles trichomycin. The minor components of the antibiotics were not considered as they may be members of a different sub-group.

The long retention time of candicidin is noteworthy because, of all of the antibiotics tested, it revealed the strongest ability to bind to the hydrophobic phase of the

TABLE III

RELATIONSHIP BETWEEN HSLC RETENTION TIME AND ANTIFUNGAL ACTIVITY OF POLYENE MACROLIDE ANTIBIOTICS

Antibiotic	*Chromophore class*	*HSLC retention time* (min)*	*M.I.C.** (μg/ml)*
Filipin	Methylpentaene	*2.6*; 3.8	5.0
Eurocidin	Pentaene	*2.8*; *3.5*	
Nystatin	Tetraene	*3.0*; 3.4	0.8–3.1
Candidin	Heptaene	*3.0*; 3.6	0.5
Amphotericin B	Heptaene	3.4	0.09–0.5
Hamycin	Heptaene	*5.0*; 7.9	0.012–0.015
Fungimycin (perimycin)	Heptaene	*5.2*; 6.1	0.07
Trichomycin	Heptaene	5.4	0.03–0.25
Mediocidin	Hexaene	6.3	0.035
Candicidin	Heptaene	*8.0*; 12.0	0.03

* For antibiotics containing more than one component, the main one is given in italics. Conditions of HSLC separation are described in Fig. 7.

** Minimal inhibitory concentration *in vitro* to *S. cerevisiae*[9].

TABLE IV

HSLC CLASSIFICATION OF HEPTAENE MACROLIDES

Class	*Antibiotics*	*HSLC retention time (min)*	*M.I.C.* (μg/ml)*
A	Amphotericin Candidin	3.0–3.6	0.25–0.5
B	Fungimycin Trichomycin Hamycin**	5.0–5.4	0.03–0.07
C	Candicidin	8.0–12	0.03

* Minimal inhibitory concentration *in vitro* to *S. cerevisiae*[9]. Approximate range of activity.
** Hamycin appears to be a member of this group although lower M.I.C. values were reported[9].

column packing and may reflect not only the antifungal potency but also the unique strong ability of candicidin to bind with enterohepatic cholesterol in man and animals[3].

CONCLUSIONS

As the present study was aimed at developing an HSLC method applicable to all classes of the polyene macrolide antibiotics, no special emphasis was placed on achieving optimum separation conditions for individual antibiotics. The resolution was also compromised in experiments yielding the separation curves shown in Fig. 7 and involving a comparison of different antibiotics under identical conditions. A considerable improvement in the resolution of particular antibiotics was made possible merely by adjusting the proportion of THF in the solvent mixture used for chromatography.

The influence of HSLC parameters other than solvent composition on the resolution of polyene antibiotics was also investigated, including temperature, column length and flow-rate. It was found that adjustment of these parameters was less effective and less practical than solvent modification.

The main drawback of the HSLC method developed for separating the polyene antibiotics appeared to be sample load capacity, which was limited to about 10 μg for the more difficult to separate antibiotics and 100 μg for others. This limitation, however, was significantly compensated by the high sensitivity, resolution power, reproducibility and speed of the method, which are essential factors in the screening for new polyene antibiotics, fermentation analysis, metabolic studies and other investigations. Because of the high antifungal activity of many polyene antibiotics, it is also possible to collect individual pure fractions of the antibiotics after HSLC separation and to evaluate them for their bioactivity.

It seems most likely that the rapid development of HSLC technology, involving the introduction of more efficient column packings, new solvent delivery systems and improved detectors, will further increase the scope of the HSLC method of separating the polyene macrolide antibiotics introduced here.

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