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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE COMPARISON OF HEPTAENE MACROLIDE ANTIBIOTICS

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SUMMARY

A rapid and efficient isocratic high-performance liquid chromatographic method for the comparison of heptaene macrolide antibiotics has been developed, and the compositions of candicidin, levorin, hachimycin (trichomycin), hamycin and aureofungin are compared. Isolation of the main components of candicidin and a stability study was carried out by means of the method.

INTRODUCTION

Within the group of antibiotics with polyene macrolide structure the heptaene macrolides represent a complex subdivision. As each of the heptaene macrolides is often a mixture of several similar components an efficient separation procedure is necessary to obtain good characterization of the compounds.

Methods using high-performance liquid chromatography (HPLC)[1,2] and the coil planet centrifuge[3] have previously been shown to be the most successful for this purpose. None of them, however, has appeared suitable for routine analysis owing to lack of efficiency[1] or long analysis times[2,3].

In this paper we present a rapid HPLC method which when applied to a number of heptaene macrolide antibiotics containing a *p*-aminoacetophenone moiety afforded good resolution of their components and also separation on a preparative scale.

EXPERIMENTAL

Apparatus

A liquid chromatograph consisting of a Gynkotek 200 pump, a Cecil 212 spectrophotometer detector and a Rheodyne 7120 injection valve with a 60-μl loop

was used. Chromatograms were recorded on a Kipp & Zonen BD-8 recorder. Retention and integration data were collected simultaneously by means of a Hewlett-Packard 3353 A laboratory data system. The UV–visible spectra were recorded on a Beckmann Acta C III spectrophotometer.

Chemicals

Acetonitrile HPLC S grade were obtained from Rathburn Chemicals (Walkerburn, Great Britain). All other solvents and reagents were obtained from E. Merck (Darmstadt, G.F.R.). The suppliers of the heptaene macrolides investigated appear in Table I.

Chromatography

For thin-layer chromatography (TLC), Kieselgel 60 F_{254} (DC-Fertigplatten, E. Merck) plates (20 × 20 cm) were used with a mobile phase consisting of chloroform–ethyl acetate (3:1). Visualization was done in UV light at 254 nm or after spraying with 1% sodium nitrite in 1 *N* hydrochloric acid followed by 0.5% naphthylethylenediamine dihydrochloride in ethanol.

In HPLC, for analytical separations, a column (150 × 4.65 mm I.D.) packed with Nucleosil 5 C_8 (5 μm), Macherey, Nagel & Co. (Düren, G.F.R.), was used. For the preparative separation a column (200 × 7.6 mm I.D.) packed with the same material was used. Both columns were packed as described earlier[4]. The efficiency of the columns expressed as the number of theoretical plates (*N*) measured on naphthalene when eluted by 80% methanol in water (capacity factor, $k' = 1.0$) at a linear solvent velocity of 1.4 mm/sec was $N = 10{,}000$ for the analytical column and $N = 16{,}000$ for the preparative column. The columns were operated at room temperature. Acetonitrile–0.05 *M* ammonium acetate buffer, pH 4.6 (37.5:62.5), was used as the mobile phase.

Test solutions

In TLC, 25 mg of the sample in question was heated for 30 min with 25 ml of 2 *N* sodium hydroxide on a steam-bath. The reaction mixture was extracted twice with 25 ml of methanol–diethyl ether (2:23). The combined organic layers were dried

TABLE I

THE INVESTIGATED *p*-AMINOACETOPHENONE-CONTAINING HEPTAENE MACROLIDE ANTIBIOTICS

Sample		*Source*
A	Candicidin	1st British Standard of candicidin. National Institute for Biological Standards and Control (London, Great Britain)
B	Candicidin	Dumex Ltd. (Copenhagen, Denmark)
C	Candicidin	S. B. Penick & Co. (N.J., U.S.A.)
D	Levorin	All-Union Research and Technological Institute of Antibiotics and Enzymes (Leningrad, U.S.S.R.)
E	Hachimycin	National Institute for Biological Standards and Control
F	Hamycin	National Institute for Biological Standards and Control
G	Aureofungin	National Institute for Biological Standards and Control

with anhydrous sodium sulphate and evaporated to dryness. The residue was redissolved in 500 μl of methanol and 10 μl were applied on the chromatoplate.

In HPLC, solutions in dimethyl sulphoxide were used for both analytical (0.1%) and preparative (0.5%) separations, and injected volumes were 5 and 60 μl respectively. In the analytical runs of the collected fractions, 60 μl were injected.

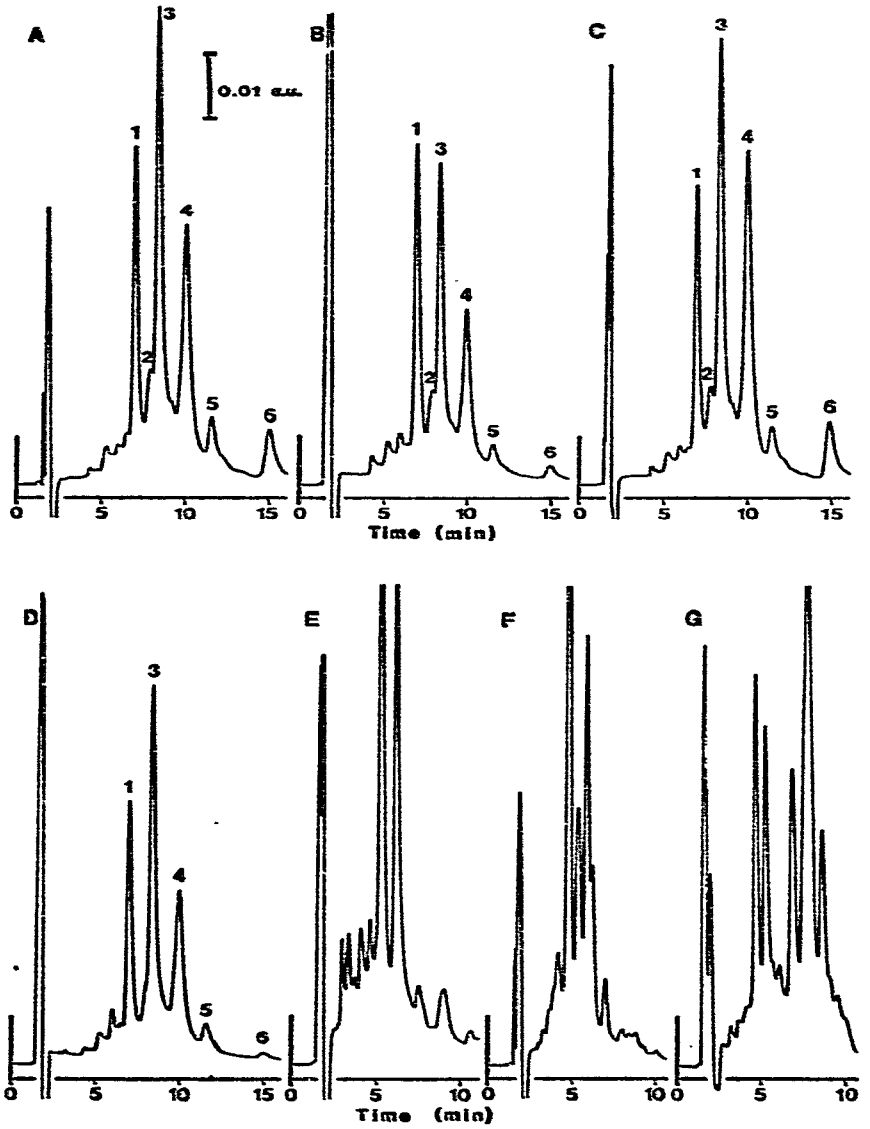

Fig. 1. Chromatograms of seven heptaene macrolide antibiotics (for identification see Table I). Support: Nucleosil 5 C_8, 150 × 4.65 mm. Mobile phase: acetonitrile–0.05 *M* ammonium acetate buffer, pH 4.6 (37.5:62.5). Solvent velocity: 1.4 mm/sec. Pressure: 8 MPa. Detection wavelength: 330 nm. Identical peak numbers in chromatograms A–D indicate components exhibiting the same retention.

RESULTS AND DISCUSSION

That the heptaene macrolides investigated in this study all contain a *p*-aminoacetophenone moiety was shown by carrying out the retro aldol condensation, extracting the liberated aromatic amine and identifying it by TLC and a colour reaction. All the samples in Table I exhibited a spot on the chromatoplate corresponding to *p*-aminoacetophenone (R_F 0.25), which upon diazotization and coupling with naphthylethylenediamine took on the same colour as the reference compound. In UV light at 254 nm aureofungin gave rise to a spot of similar size (R_F 0.35) which could not be diazotized and which is assumed to correspond to N-methyl-*p*-aminoacetophenone.

For the HPLC separation, Nucleosil 5 C_8 among several reversed-phase materials was found suitable for separating the heptaene macrolides. Acetonitrile appeared to be superior to methanol, as the modifier in the aqueous mobile phase, but in order to achieve the highest efficiency it was essential to adjust the ionic strength and the pH. An increase in the buffer ionic strength caused an overall increase in the eluting power of the mobile phase, and it was found that at buffer concentrations below 0.01 *M* the macrolides could not be eluted. The optimal pH was determined to range between 4 and 6, and an 0.05 *M* ammonium acetate buffer of pH 4.6 was chosen for the separation, although the effect of a 0.05 *M* phosphate buffer of the same pH value was equivalent.

Table I lists the heptaene macrolide antibiotics which were compared by the HPLC method (Fig. 1). Three samples of candicidin (A, B and C) of different origin contain the same components (as indicated by retention time). The 1st British Standard (A) is chromatographically identical to the Penick sample (C), in accordance

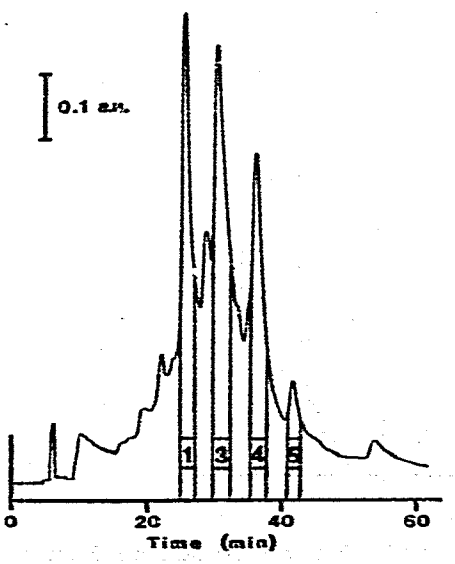

Fig. 2. Preparative separation of candicidin, Dumex. The isolated fractions are indicated. Support: Nucleosil 5 C_8, 200 × 7.6 mm. Solvent velocity: 0.5 mm/sec. Pressure: 6.5 MPa. Other conditions as in Fig. 1.

with the fact that the starting material for A was produced by Penick[5]. The Dumex sample (B) has a slightly different relative ratio of the components. Levorin (D) and candicidin are identical, as has been shown earlier[2,3,6]. Hachimycin (trichomycin) (E), hamycin (F) and aureofungin (G) are all different and they differ from candicidin and levorin as well.

Attempts were made to isolate the main heptaene components of candicidin by freeze-drying collected fractions, but they are unstable when redissolved. Therefore

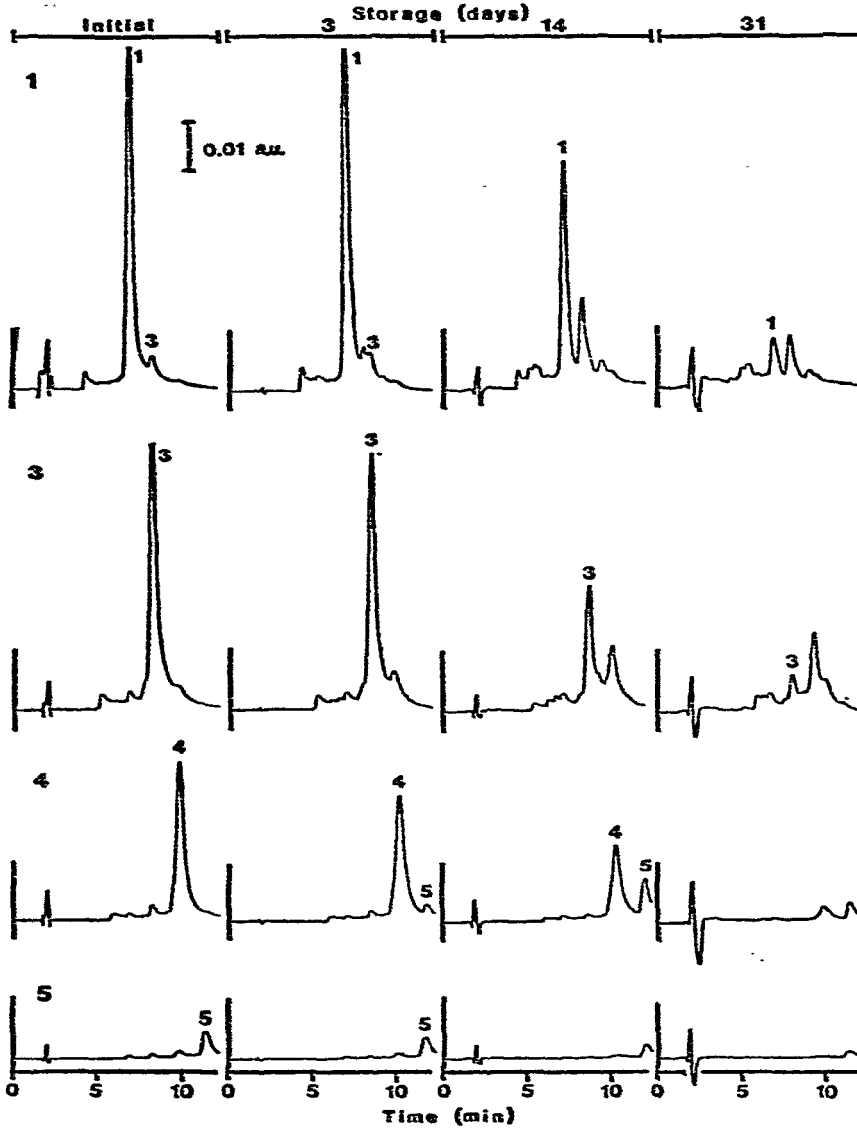

Fig. 3. Stability of the four isolated fractions (Fig. 2) as a function of storage time. The peak numbers correspond to those in Fig. 1. Conditions as in Fig. 1.

the stability of the single components in solution (the mobile phase in the HPLC system) was studied. Fractions from the preparative column (Fig. 2) were collected and stored in 5 ml screw cap vials at ambient temperature in diffused daylight. The UV–visible spectra of each of the isolated components recorded in the mobile phase were identical for fractions 1, 3 and 4 with maxima at 401, 379 and 360 nm. The spectrum of fraction 5 exhibited maxima at 405, 382 and 361 nm, which might indicate an "all *trans*" isomer.

The degradation of the single components was followed for 31 days (Fig. 3). It is seen that the chromophore is gradually destroyed, and in fractions 1, 3 and 4 new peaks appear after some time. Finally these peaks also disappear. By careful study of the retention times it was found that for fractions 1 and 3 none of the emerged peaks was identical with any one peak in the original sample. For fraction 4, however, retention times indicate that the primary conversion product is identical to component 5. This indicates that not all the many components found in the heptaene macrolide antibiotics are due to partial degradation.

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