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SEPARATION OF AROMATIC HEPTAENE ANTIBIOTICS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Thin-layer chromatography and high-performance liquid chromatography have been used to separate and characterize representatives of the aromatic heptaene group of antifungal antibiotics. Most antibiotics were complex mixtures that could be placed in well defined groups. The identity of candicidin and levorin was confirmed in one group. Aureofungin, DJ400 and hamycin shared many components in common in the second group. The proportion of the individual components of lucknomycin and trichomycin was markedly different from each other and from the antibiotics in the other two groups. Lucknomycin stood out as the most homogeneous of the antibiotics examined.

INTRODUCTION

The aromatic heptaene antibiotics are a group of antifungal antibiotics possessing a large lactone ring containing a hydroxylated portion and a characteristic system of seven conjugated double bonds, an amino sugar (mycosamine or perosamine) and an aromatic entity (*p*-aminoacetophenone or N-methyl-*p*-aminoacetophenone). Most of these antibiotics are available only as complex mixtures[1]. Only two[2,3] of the previous high-performance liquid chromatographic (HPLC) studies[2–5] have attempted a comprehensive comparison of the heptaenes available.

In order to determine the identity of the recently isolated and purified heptaene antibiotic lucknomycin[6], it has been compared with other antibiotics in this group by HPLC and thin-layer chromatography (TLC).

EXPERIMENTAL

Materials

The aromatic heptaene antibiotics examined are listed with their suppliers in Table I. Pre-coated chromatoplates silica gel 60 HPTLC, 100 × 200 mm (Art 5641, E. Merck), acetonitrile (HPLC grade, Rathburn), ammonium pentaborate (Sigma) and analytical-grade solvents and reagents (BDH) were used.

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TABLE I

THE AROMATIC HEPATAENE ANTIBIOTICS EXAMINED

Antibiotic	*Source*
Aureofungin	Hindustan Antibiotics, Pimpri, India
Candicidin	Dumex, Copenhagen, Denmark
Candicidin	S. B. Penick Corp., New Jersey, U.S.A.
DJ400	Schering, Berlin, F.R.G.
Hamycin	Hindustan Antibiotics Ltd., Pimpri, India
Levorin	U.S.S.R. Research Technological Institute for Antibiotics and Enzymes for Medical Use, Leningrad, U.S.S.R.
Lucknomycin	UCB, Brussels, Belgium
Trichomycin	National Institute of Health, Tokyo, Japan

TLC

Plates were sprayed with 0.1 *M* ammonium pentaborate then dried at 140°C for 60 min. The mobile phase consisted of the lower phase of a mixture of chloroform–methanol–dioxan–glacial acetic acid–0.1 *M* ammonium pentaborate (33:38:9:1:19).

The filter-paper-lined chromatography tank was equilibrated with the mobile phase for 24 h at room temperature, then 3 h at 37°C. The antibiotics were dissolved in the lower phase of a mixture of chloroform–methanol–borate buffer (pH 8.3) (4:4:2), 3 mg ml^{-1} except lucknomycin 1.5 mg ml^{-1}, and 8-μl aliquots were applied to the surface of the plate with a micro-syringe. The plates were developed over a distance of 15 cm in a fully saturated tank at 37°C, then they were air-dried, sprayed with anisaldehyde–sulphuric acid reagent (5%, v/v, anisaldehyde and 5%, v/v, sulphuric acid, sp.gr. 1.84, in methanol) and heated 100°C for 5 min.

For biological detection, air-dried plates were covered with a 4 mm thick layer of candicidin assay agar[7] inoculated with a suspension of *Saccharomyces cerevisiae* NCYC 10716 and incubated at 32°C for 18 h. Antifungal activity was revealed as a clear zone of inhibition of growth.

Individual components were located on the plate under ultraviolet light, and the appropriate area of silica gel was scrapped off and extracted with methanol. The resulting suspension was passed through a low-volume filter unit, 0.45 μm APD (Millex HV_4, Millipore) then examined by HPLC.

HPLC

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 (Model CE272, Cecil Instruments) fitted with a 75-μl flow-through cell. A presaturation column, Spherisorb S10, ODS1 (100 × 4.6 mm I.D.) was placed between the solvent mixing chamber and the injector; a Spherisorb S5, C_8 column (200 × 4.6 mm I.D.) was used for the analytical separation. The mobile phase was filtered through a glass microfibre filter and degassed prior to use. It consisted of acetonitrile, 0.1 *M* sodium dodecyl sulphate and 0.005 *M* ammonium acetate–succinic acid buffer (pH 4.6). The selected gradient elution profile gave an exponential increase (exponent = 2) in acetonitrile concen-

tration from 40 to 46% v/v in 30 min. The flow-rate was 0.5 ml min^{-1}. The antibiotics were dissolved in dimethyl sulphoxide 2.8 mg ml^{-1}, except lucknomycin 1.4 mg ml^{-1}, diluted 1:10 with methanol and injected through a Rheodyne injector (Model 7125) with a 20-μl fixed loop. The eluent was monitored at 380 nm, sensitivity 0.2 a.u.f.s. The relative composition of the samples was determined by normalisation of peak areas measured using a computing integrator (Model SP 4270, Spectra Physics); it was assumed that the heptaene components have the same absorbance at 380 nm.

RESULTS AND DISCUSSION

TLC

TLC revealed the complex nature of the aromatic heptaene antibiotics examined, lucknomycin appeared by far the most homogeneous. The hR_{St} values of the major components in the antibiotics are given in Table II, and a typical chromato-

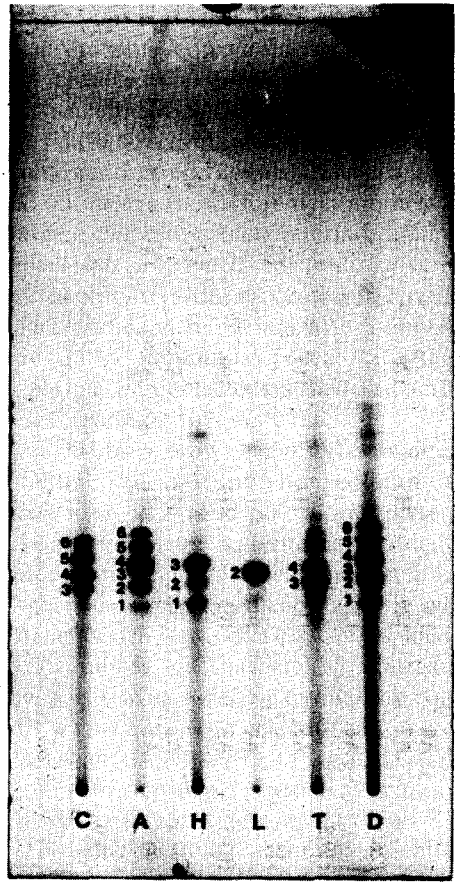

Fig. 1. Thin-layer chromatogram of aromatic heptaene antibiotics detected with anisaldehyde–sulphuric acid reagent. Silica gel 60 HPTLC; lower phase of mixture chloroform–methanol–dioxan–glacial acetic acid–0.1 *M* ammonium pentaborate (33:38:9:1:19). C = Candicidin; A = aureofungin; H = hamycin; L = lucknomycin; T = trichomycin; D = DJ400.

TABLE II

THE hR_{St} VALUES OF THE COMPONENTS OF THE AROMATIC HEPTAENE ANTIBIOTICS

hR_{St} values calculated from thin-layer chromatograms of aromatic heptaene antibiotics. Silica gel 60 HPTLC, lower phase of mixture chloroform–methanol–dioxan–glacial acetic acid–0.1 *M* ammonium pentaborate (33:38:9:1:19).

$$hR_{St} = \frac{\text{distance of sample spot from origin}}{\text{distance of reference spot (L2) from origin}} \times 100.$$

Candicidin		*Aureofungin*		*Hamycin*		*Lucknomycin*		*Trichomycin*		*DJ400*	
Ident. No.	hR_{St}	*Ident. No.*	hR_{St}	*Ident. No.*	hR_{St}	*Ident. No.*	hR_{St}	*Ident. No.*	hR_{St}	*Ident. No.*	hR_{St}
										D11	177
										D10	170.5
								T10	167.5		
		A9	165	H6	164.5					D9	163.5
						L4	157	T9	157.5	D8	156
		A8	137								
		A7	129	H5	129					D7	128.5
C7	126							T8	125.5		
		A6	122							D6	121
C6	119.3							T7	118		
		A5	115	H4	117			T6	114.5	D5	115
C5	111					L3	111.8	T5	110.5		
		A4	108							D4	108
C4	103.5	A3	105	H3	106			T4	102.5	D3	103.8
						L2	100				
C3	97.5	A2	97.1	H2	97.5			T3	94.6	D2	95.8
C2	90.4	A1	88	H1	87.6	L1	87.2	T2	88	D1	85.6
								T1	78.4		
C1	74.9										

gram is shown in Fig. 1. The antibiotics reacted to give distinctive colours with the anisaldehyde spray as follows: candicidin and levorin, dark pink; lucknomycin, mauve; aureofungin, DJ400 and hamycin, grey-green; and trichomycin, dark green. Individual components in aureofungin, DJ400 and hamycin were different colours: A1, D1, H1, green; A2, C3, D2, H2, pink; A3, D3, H3, green; A4, D4, pink; A5, D5, mauve; A6, D6, grey. Anisaldehyde–sulphuric acid spray is used to identify carbohydrates separated by TLC[8]. Different colour reactions of the components of aureofungin, DJ400 and hamycin suggest that these components differ by the presence of different sugars attached to the aromatic heptaene moiety, although to date only two different sugars, mycosamine and its isomer perosamine, have been found in the aromatic heptaene antibiotics.

Bioautography showed that all the major components had antifungal activity. Individual components were collected from the silica plate to be extracted and examined by HPLC. Attempts to compare the major components as revealed by TLC using HPLC were abandoned when it was found that some of the components degraded during the extraction process and that the extracted material was extremely unstable. HPLC showed lucknomycin to consist of 70% (peak 13) and 22% (peak 17), examination of the freshly extracted major component (hR_{St} 100) showed 35% (peak 13) and 61% (peak 17), 60 min later the composition of this extract had changed to 23% (peak 13) and 71% (peak 17). The instability of candicidin[3], lucknomycin[6] and DJ400[9] has been reported previously.

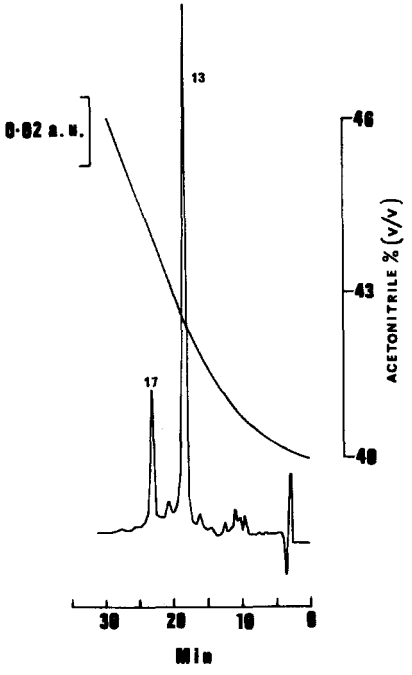

Fig. 2. Chromatogram of lucknomycin, showing gradient profile of increase of acetonitrile concentration in eluent. Peak identification and chromatographic conditions as in Table III.

TABLE III

COMPOSITION OF THE AROMATIC HEPTAENE ANTIBIOTICS

Composition (%) of the aromatic heptaene antibiotics based on the measurement of peak areas of the components separated by HPLC. Major components (> 10%) boxed. Spherisorb C_8 column (200 × 4.6 mm I.D.); eluent, 0.1 *M* sodium dodecyl sulphate, 0.005 *M* ammonium acetate–succinic acid buffer (pH 4.6), acetonitrile (40% v/v increasing exponentially, exponent = 2 to 46% v/v in 30 min); flow-rate, 0.5 ml min^{-1}; measured at 380 nm.

	Peak No.											
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Capacity factor (*k'*)	0.85	1.01	1.31	1.70	1.96	2.08	2.44	2.57	2.82	3.22	3.36	3.73
Candicidin 1	–	0.49	–	–	–	–	–	–	–	–	–	1.00
2*	–	2.01	0.29	–	0.68	–	0.68	–	–	–	0.42	0.92
Levorin	–	1.26	–	–	–	–	–	–	–	–	0.80	–
Aureofungin 1*	0.14	1.00	0.25	–	*14.19*	6.82	*10.58*	–	3.68	–	2.23	9.97
2	1.32		0.77	–	*10.17*	4.21	*12.21*	–	2.44	–	1.40	7.07
DJ 400	–	1.53	0.49	1.53	*21.53*	*11.27*	*16.09*	–	2.01	–	0.33	6.87
Hamycin 1*	0.16	1.08	0.24	3.24	*23.62*	*12.00*	*11.69*	–	*26.96*	–	6.93	2.88
2*	1.77	1.30	0.29	0.64	*43.84*	*19.23*	*14.10*	–	*16.65*	–	2.19	–
Lucknomycin*	–	–	–	–	1.75	2.82	1.45	–	0.13	–	1.19	–
Trichomycin*	0.13	5.61	5.55	4.23	2.19	3.59	–	*30.61*	7.23	*19.58*	9.42	–

* Chromatogram shown.

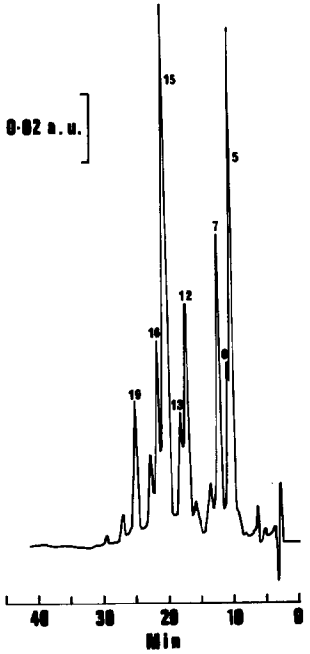

Fig. 3. Chromatogram of aureofungin, sample 1. Peak identification and chromatographic conditions as in Table III.

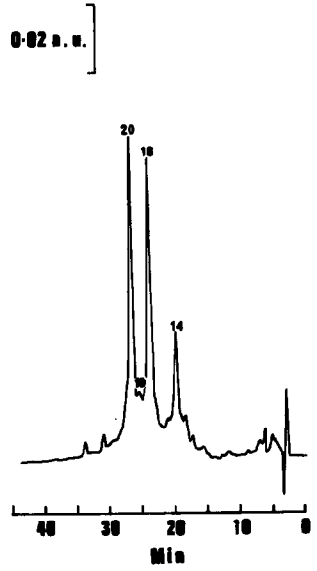

Fig. 4. Chromatogram of candicidin, sample 2. Peak identification and chromatographic conditions as in Table III.

13	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*
3.92	4.33	4.50	4.87	5.06	5.42	5.71	6.05	6.16	6.89	7.14	7.76	9.25	10.36
–	*13.06*	4.08	–	–	*31.89*	7.59	*30.39*	–	1.13	1.58	7.82	–	–
1.43	*13.49*	2.47		–	*34.52*	5.59	*35.82*	–	–	–	–	–	–
–	*23.29*	1.80		–	*41.63*	3.92	*24.28*	0.14	–	2.23	0.65	–	–
6.38	–	*24.59*	7.55	4.21	–	6.28	–	–	0.88	0.24	–	–	–
5.45	–	*21.33*	*13.15*	5.60	–	*10.59*	–	–	1.78	0.60	–	–	–
4.83	–	*12.54*	*11.25*	4.12	–	5.01	–	–	0.58	–	–	–	–
2.69	–	3.42	1.09	2.19	–	1.70	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–
69.88	–	1.12	–	*21.66*	–	–	–	–	–	–	–	–	–
1.99	2.23	2.22	–	2.48	–	–	–	–	–	–	–	1.81	0.83

HPLC

The method developed confirmed the heterogeneous nature of the aromatic heptaene antibiotics and revealed up to 26 different components in the antibiotics examined (Table III). The best separation obtained utilised a gradient elution system as shown in Fig. 2. The peaks have been numbered and the major components marked in the chromatograms to assist in recognising those components that occur in more than one antibiotic. Chromatograms of separations of lucknomycin, aureofungin, candicidin, hamycin and trichomycin are shown in Figs. 2–7. The two samples of candicidin from different sources were very similar to each other and to levorin, confirming earlier reports that candicidin and levorin are identical, differing only in the relative composition[1–3]. Two different samples of the same antibiotic hamycin showed a marked difference in their relative composition: sample 1 contained 23.62% peak 5 and sample 2 contained 43.84% (Figs. 5 and 6).

Aureofungin, DJ400, hamycin, lucknomycin and trichomycin shared a number of minor components. Aureofungin, DJ400 and hamycin also shared a number of major components. Similarity between the HPLC profiles of aureofungin, DJ400, hamycin and trichomycin have been noted before[2]. Detailed examination of the relative composition can differentiate aureofungin and hamycin, DJ400 appeared to be a mixture of the two antibiotic types. The proposition of the individual components of lucknomycin and trichomycin was markedly different from each other and from the other aromatic heptaene antibiotics examined.

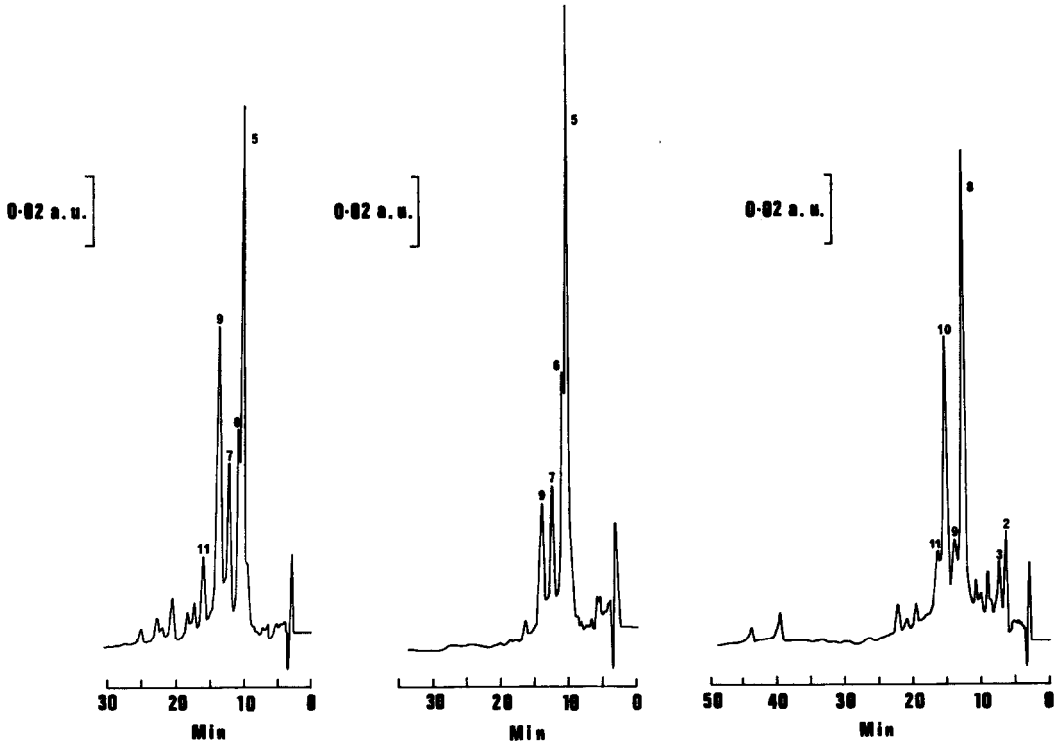

Fig. 5. Chromatogram of hamycin, sample 1. Peak identification and chromatographic conditions as in Table III.

Fig. 6. Chromatogram of hamycin, sample 2. Peak identification and chromatographic conditions as in Table III.

Fig. 7. Chromatogram of trichomycin. Peak identification and chromatographic conditions as in Table III.

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