CHROM. 4996

Pyrolysis–gas chromatography of polyene antifungal antibiotics: the nature of candicidin, levorin and trichomycin

The polyene antifungal antibiotics are labile substances which possess a macrocyclic skeleton containing a conjugated system of up to seven double bonds, together with other substituents[1]. The highest antifungal activity is exhibited by the heptaene compounds. These are also the most complex chemically, with molecular weights in the range 900–1300, and most difficult to purify, and their complete structures are as yet unknown. Three heptaene preparations, candicidin from *Streptomyces griseus*, levorin from *S. levoris* and trichomycin from *S. hachijoensis*, isolated respectively in U.S.A., U.S.S.R. and Japan, have each been separated into several components by counter-current distribution[2–4]. Studies based on such separations, together with other chromatographic and spectroscopic evidence and biological potency data, suggest that the major components in levorin and candicidin may be identical[2] and those in trichomycin and candicidin may be the same[3]. KALÁSZ *et al.*[3] were also unable to differentiate between an antibiotic S-515 from *S. levoris* and candicidin and concluded that they are identical. S-515 may be considered as a levorin sample. Exhaustive comparison of the three substances is hampered by their degradation during prolonged handling. Detailed, but incomplete, proposals for chemical structure have only been published for trichomycin A (ref. 5).

BRODASKY[6] demonstrated the application of pyrolysis–gas chromatography, a fingerprinting technique established in the field of polymer chemistry[7], to differentiation and characterisation of some antibiotics. His results also indicated the sensitivity of the pyrolysis chromatograms (pyrograms) to small changes in structure and stereochemistry. We have, therefore, investigated the behaviour of some polyene antibiotics after low- and high-temperature pyrolysis with the particular object of studying the identity of candicidin, levorin and trichomycin. This paper describes our results.

Materials and methods

Antibiotics. The W.H.O. 1st International Standards of nystatin and amphotericin B were used. Candicidin was provided by S.B. Penick and Co., New York (Batch No. 8461-NJF-1); one sample (Batch No. 684-NKF-1) was obtained through Pharmax Ltd., Dartford. Levorin, including samples of five separate production batches for which all the data in Table I were supplied, was generously donated by Dr. W. O. KUHLBAKH, Leningrad. Trichomycin (Lot No. 001643I) was given by Fujisawa Pharmaceutical Co. Ltd., Osaka. As far as possible all the samples were stored at —20° and in the dark to prevent decomposition. Solutions (about 10 mg/ml) were prepared in dimethylformamide or water immediately before use. The solubility of some samples in water was increased by addition of a trace of sodium hydroxide solution.

Pyrolysis. (a) Low temperature. The solution (6 μl containing about 60 μg antibiotic) was applied to the tip of a solids injector (Scientific Glass Engineering Pty. Ltd., London N.20) and the solvent evaporated. The solid residue was held in the injection block of the gas chromatograph for 10 sec at 380° $\pm$ 10°, then the needle withdrawn. Glass liners fitted in the block were changed when contaminated.

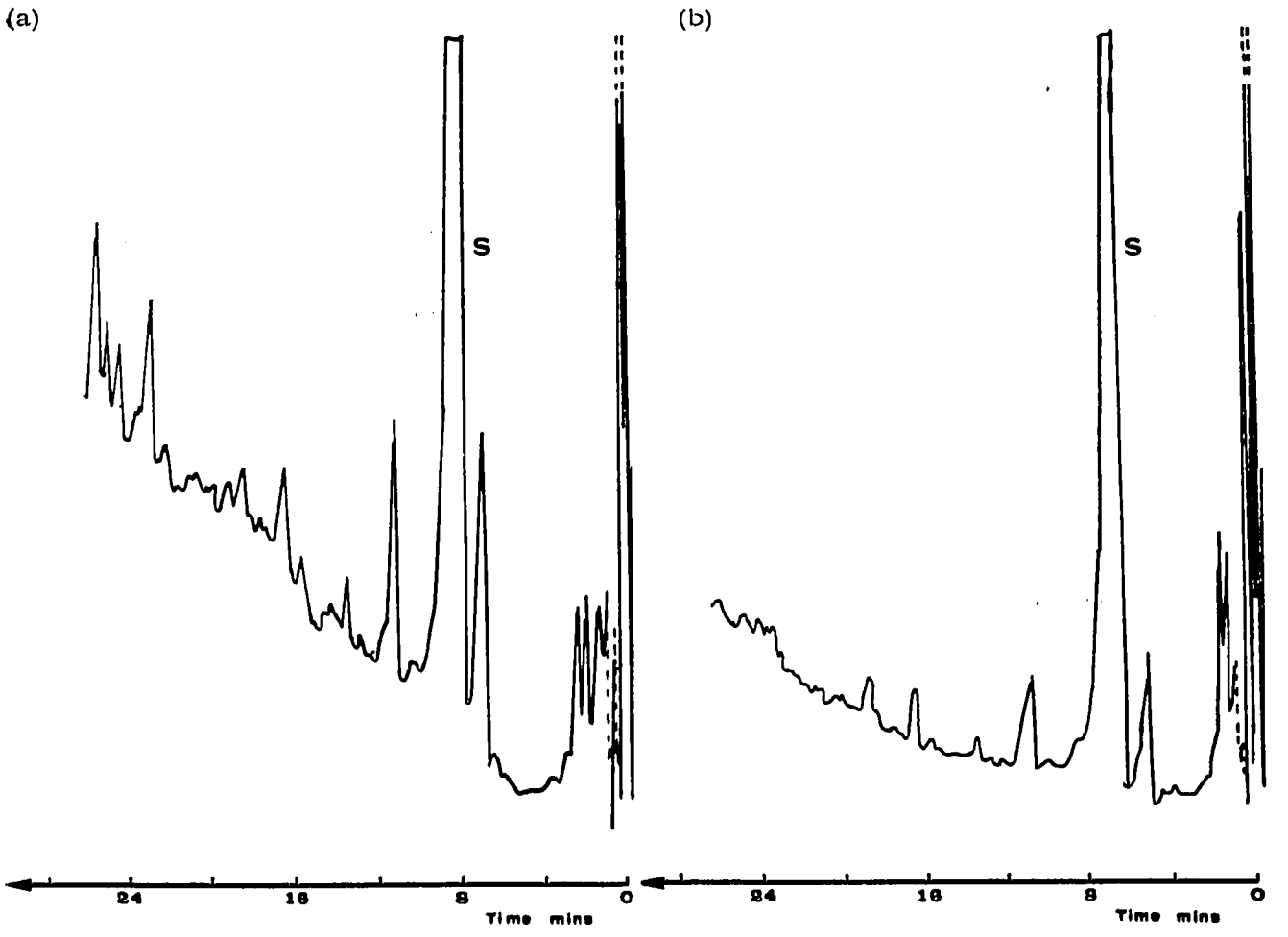

Fig. 1. Chromatograms of (a) nystatin and (b) amphotericin B after heating for 10 sec at 380°. Solid obtained by evaporation from dimethylformamide solution. Column: 1 m × 2.2 mm I.D. stainless steel containing 5 % FFAP on Chromosorb G AW-DMCS. Nitrogen carrier at 15 ml/min. Programme: 4 min at 75°, then 6°/min to 200°, then isothermal.

(b) High temperature. The solution was coated on the filament of a pyrolyser unit (Bodenseewerk Perkin-Elmer and Co., GmBH, Überlingen/See) and solvent evaporated. The unit was fitted to the chromatograph injection block and current applied so that the solid was heated for 10 sec at about 900°.

Gas chromatography. Gas chromatography was performed with a Perkin-Elmer F11 instrument equipped with a flame ionisation detector. Nitrogen was used as carrier gas at 15 ml/min. Several stationary phases were investigated and best results obtained with a 1 m × 2.2 mm I.D. stainless steel column packed with 5 % FFAP on Chromosorb G AW-DMCS. Pyrolysis products were separated by a temperature programme of 4 min at 75°, then increasing at 6°/min to 200°. The total run was about 30 min.

Results and discussion

Preliminary experiments indicated the usefulness of FFAP, a modified Carbowax 20M, as liquid phase for the separation of pyrolysis products, in accord with the

findings of BRODASKY[6]. We also found that low-temperature pyrolysis generally resulted in more intense and characteristic chromatograms than pyrolysis at high temperature.

Figs. 1–3 reproduce chromatograms obtained with the five antibiotics studied, after pyrolysis for 10 sec at 380°. Slight differences in retention time of peaks for the different samples reflect small variations in experimental conditions, such as temperature and carrier gas flow, over several months.

Fig. 1 shows that nystatin and amphotericin B give pyrograms which differentiate the two substances. The large peak, S, arises from traces of solvent, dimethylformamide, occluded in the solid during evaporation of the sample before pyrolysis.

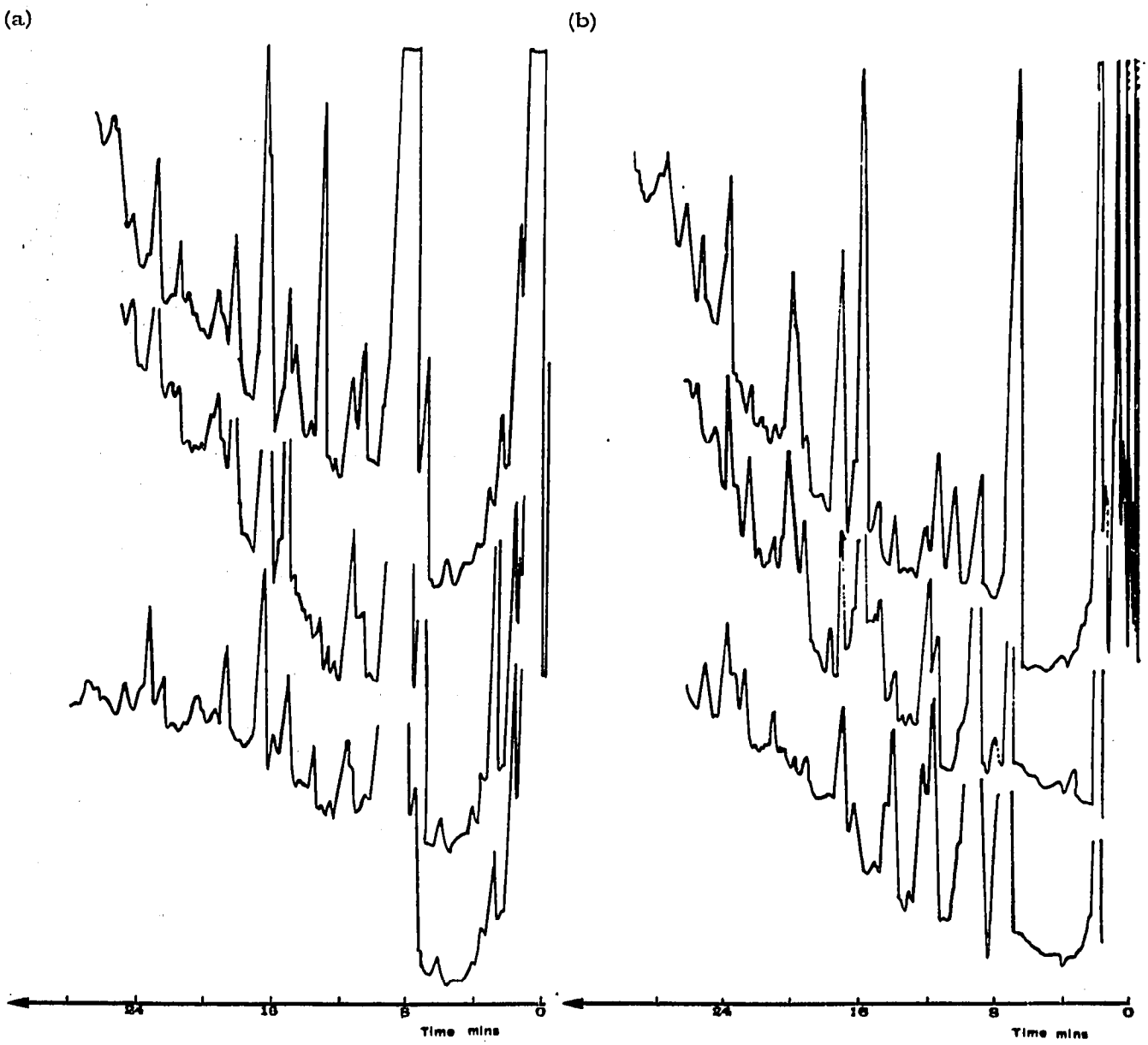

Fig. 2. Chromatograms of levorin (upper), candicidin (middle) and trichomycin (lower) after heating for 10 sec at 380°. Conditions as in Fig. 1. Solid obtained by evaporation from (a) dimethylformamide solution and (b) aqueous solution.

Its identity is confirmed by the behaviour of dimethylformamide under the same programming conditions. Although their complete structures are unknown, both nystatin[8] and amphotericin B[9] contain 38-membered lactone rings with the same substituents at some positions. Common peaks in the two pyrograms probably arise from fragments derived from identical regions in the two molecules.

Fig. 2 shows pyrograms from samples of candicidin, levorin and trichomycin: all differ from those in Fig. 1. They derive from solid obtained by evaporation (a)

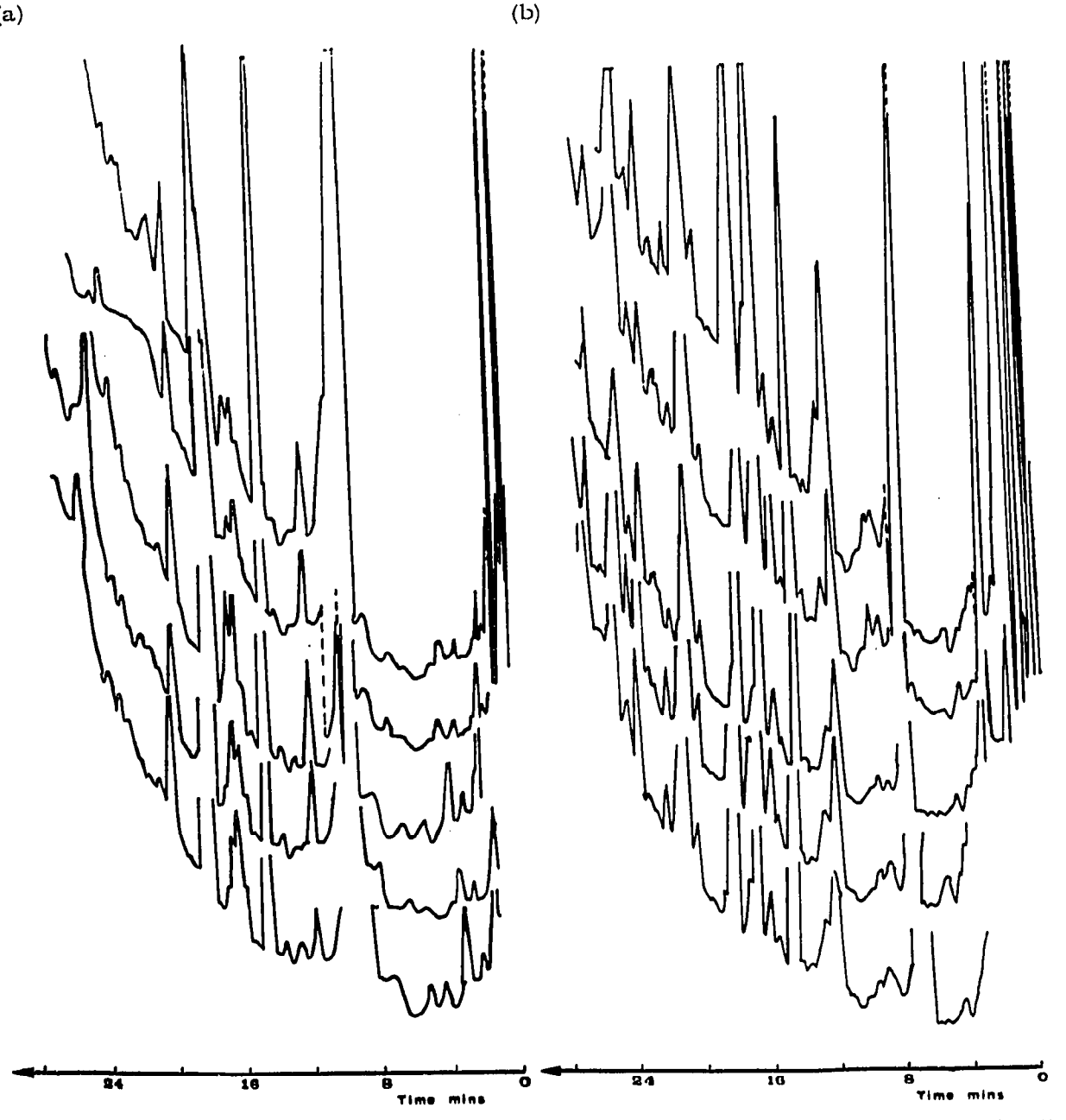

Fig. 3. Chromatograms of five samples of levorin after heating for 10 sec at 380°. Conditions as in Fig. 1. Solid obtained by evaporation from solution in (a) dimethylformamide and (b) water to which a trace of sodium hydroxide was added. Batch number (from top): (a) 10869, 12669, 16069, 11869, and 11169, and (b) 11869, 11169, 12669, 16069, and 10869.

from dimethylformamide solution and (b) from aqueous solution. In contrast to the results in Fig. 1, the patterns obtained from candicidin and levorin are in almost complete agreement. Since more than 35 peaks can be distinguished and their relative intensities are similar, this is good evidence for close structural identity of the two antibiotics.

The pyrograms from trichomycin contain essentially the same peaks but show greater quantitative differences: larger volumes of trichomycin solution in dimethylformamide were needed to give traces comparable in intensity with those from 6 μl solutions of the other preparations.

Pyrograms from solutions left exposed to air and light for up to four days showed changes in the relative intensities of a few peaks, compared with those from fresh solutions, but were basically unaltered.

Candicidin, levorin and trichomycin eliminate *p*-aminoacetophenone when treated with alkali[1,3,4]. Pyrograms from these substances, but not nystatin or amphotericin B, contain a peak corresponding to that from *p*-aminoacetophenone. This peak is observed after about 50 min on temperature-programmed analyses but is more conveniently obtained in approximately 25 min by isothermal analysis at 200°.

All five antibiotics examined contain one residue of the amino-sugar, mycosamine. No authentic sample of this was available but pyrograms obtained with glucosamine and galactosamine indicate that such molecules give rise to a number of fragments and no single, major, peak can be considered diagnostic. This is consistent with lower stability and less specific cleavage of a sugar unit compared with an aromatic one and may be likened to the fragmentation patterns of similar molecules observed by mass spectrometry.

Fig. 3 shows pyrograms obtained with five batches of levorin and are from solid after evaporation of (a) a dimethylformamide solution, and (b) an aqueous solution to which a trace of sodium hydroxide was added (to increase the solubility of the samples). Control experiments with candicidin and trichomycin showed that addition of alkali resulted in more intense pyrograms without affecting their composition.

At least thirty peaks are common to the traces in Fig. 3 although minor differences exist between the samples, for example small peaks are not always present. Each pyrogram contains the same four major peaks but their relative intensities vary. The pyrograms also differ according to the solvent originally used but this difference is constant between the samples.

Chromatograms obtained after pyrolysis of samples at 900° for 10 sec confirmed the results described above. They were weaker and contained fewer peaks, presumably reflecting greater thermal breakdown, but were otherwise essentially the same.

It is known that the components of the levorin A complex vary in their biological activities[10] and that variations in composition occur between levorin samples obtained from different strains of *S. levoris*[11]. Quantitative changes in the pyrograms in Fig. 3 probably reflect small changes in composition from sample to sample. The relationship between the pyrogram of a sample and its biological potency is, however, complex whereas that between UV absorption and potency appears, from Table I, to be qualitatively simple. The intensity of UV absorption may be considered as an indication of the heptaene content of the sample.

The pyrograms in Fig. 1 differentiate nystatin and amphotericin B from each other and from the other three antibiotics (Fig. 2). This distinction is supported by

TABLE I

SOME ANALYTICAL DATA SUPPLIED FOR SAMPLES OF LEVORIN

	Batch No.				
	10 869	*11 169*	*11 869*	*12 669*	*16 069*
Biological activity, units/mg	42 100	33 600	40 800	37 800	37 200
Specific absorption					
at 342 nm	418	300	389	337	325
at 360 nm	520	415	470	455	447
at 380 nm	702	560	680	630	620
at 402 nm	605	480	570	538	524

such chemical evidence as is available. On the other hand, the pyrograms from candicidin, levorin and trichomycin (Fig. 2) all contain similar peaks although those from trichomycin are quantitatively less like the others. The differences between candicidin and levorin are no greater than those between the five levorin samples (Fig. 3).

These observations can be explained on the basis that all the samples used to obtain Figs. 2 and 3 are mixtures with similar, if not identical, components. Small quantitative differences in composition of the levorin samples give rise to the observed variations in biological potency (Table I) and in the pyrograms (Fig. 3). Fig. 2 suggests a similar difference between levorin and candicidin and a more pronounced difference between these and trichomycin, although the main component may be identical in all three. This conclusion supports and extends the chromatographic and other evidence cited in the introduction.

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