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Analysis of Candicidin and Related Polyene Antibiotics by Means of the Coil Planet Centrifuge

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In considering the need for international standards for candicidin and trichomycin,[1] the WHO Expert Committee on Biological Standardization was concerned by a claim that the two materials were essentially the same.[2] Collaborative studies of samples of candicidin and trichomycin being considered for use as international standards failed to distinguish one from the other on the basis of ultraviolet and infrared spectroscopy, chromatography and nuclear magnetic resonance and mass spectroscopy. Biological studies of relative activities against a range of sensitive organisms suggested, however, that the materials were different. Counter-current distribution studies, which had formed the basis of the original report of their near identity, were not considered to be satisfactory because of the necessary duration (several days) of the experiment and the known instability of these polyene antibiotics.

The use of the coil planet centrifuge, which allowed counter-current separations to be carried out on a micro-scale, was described by Ito *et al.*[3] This method was applied in our laboratories to an examination of candicidin and trichomycin, but methods of detection were insufficiently sensitive to allow quantitative analysis and the PTFE tubing used was permeable to the solvents used, so that only encouraging but indefinite results were obtained. Later modifications[4] of the apparatus to a continuous process were also unsatisfactory in our hands. The further development of this technique and equipment has, however, allowed unequivocal results to be obtained. By identifying and controlling a number of previously uncontrolled variables, such as temperature, rotational speed and vibration, a procedure has been developed by Sutherland and Sharpe[5] that allows reproducible separations to be carried out and a theoretical treatment of the data allows the calculation of partition coefficients of the separated compounds.

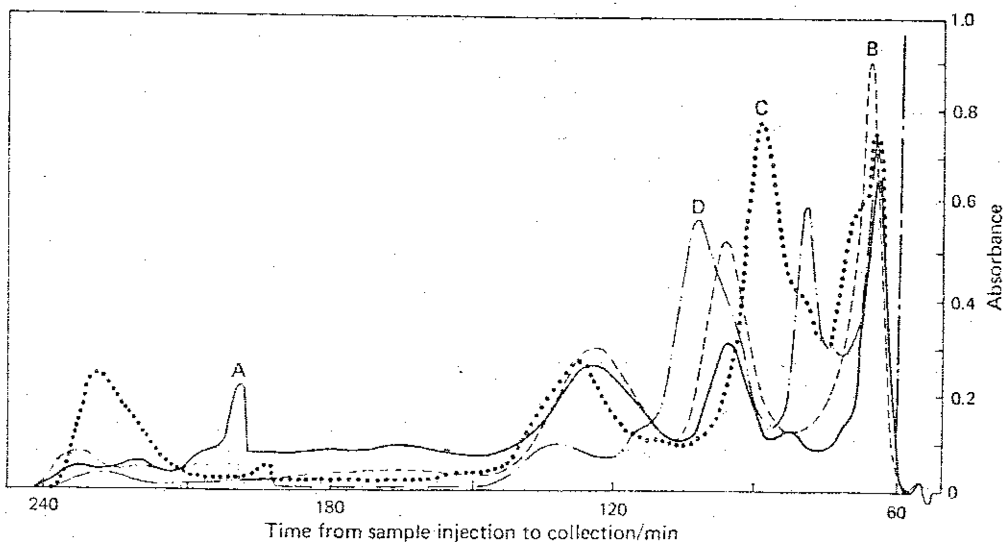

Fig. 1. Separation of four polyene antibiotics using the coil planet centrifuge. Coil volume, 110 ml; sample load, 600 μg; solvent system, chloroform - methanol - borate buffer (4+4+3). Material completely soluble in mobile phase ($P=1.0$) would be eluted at 60 min. A, Candicidin; B, levorin; C, trichomycin; and D, hamycin.

The apparatus consists of a number of interconnected separation columns that are constrained to rotate in a planetary fashion. In this way, tubes to and from the column do not twist, so that rotating seals are unnecessary. Each column is a helically wound tube that contains two immiscible solvent phases. The light phase is pumped through the columns while the heavy phase is held stationary under the opposing action of the inertia forces from the rotating acceleration vector and the drag forces from the mobile phase flow.

A sample injected with the mobile phase will therefore partition between the retained stationary phase and the pumped mobile phase. Sample constituents retained in the column in the latter stages of a separation can be eluted quickly by pumping the heavier phase as the mobile phase.

Using the system 4 + 4 + 3 chloroform - methanol - borate buffer (pH 8.2), a separation of candicidin components can be completed in 4 h, although the components of most interest are recovered in periods varying from 1 to 2½ h. In this period, separations were achieved that would require 1 000–2 000 transfers in a conventional counter-current procedure. Fig. 1 shows separations obtained using the candicidin and trichomycin samples; levorin and hamycin are included for comparison. Spectrophotometric examination of peak materials showed these to be polyene in nature. Fig. 2 shows the distribution of biological activity against *Saccharomyces cerevisiae* in relation to absorbance at 381 nm. Mixtures of candicidin and trichomycin were run and found to give the profile expected. The main biologically active peak of candicidin was collected and re-run after concentration; a single peak was obtained.

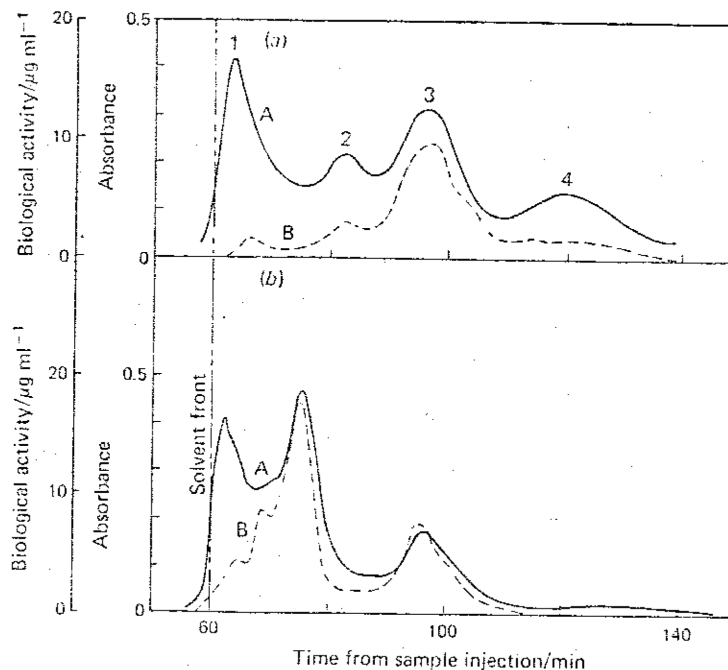

Fig. 2. Comparison of absorbance and biological activity for candicidin and trichomycin: (*a*) candicidin, 0.3 ml at 1 mg ml^{-1}; and (*b*) trichomycin, 0.3 ml at 1 mg ml^{-1}. A, Absorbance; and B, biological activity.

From the rate of movement of a peak, and the known proportions of the two immiscible phases, a value, P, can be calculated that represents the proportion of peak material in the mobile phase after correction to equal volumes. This value characterises the peak material as does K, the partition coefficient; the areas included by the various peaks would represent the proportion of that component if the absorptivity of each component was the same. Absorptivities have not yet been determined, but the proportional compositions have been calculated on the assumption that they are identical with that of the mixture, as a first approximation. Table I shows the values obtained for the sample of candicidin being considered as

possible WHO reference material, for the working standard of candicidin for the USA Food and Drug Administration and for seven different batches of candicidin from two different sources. The variation within the seven batches in the value of P for a given peak is shown as a range which varied approximately $\pm 5\%$ about the mean. The variation in proportional composition was greater, but it appeared from this table that the proposed international reference preparation (PIRP) was a representative sample of currently available batches of candicidin. The separation of the components listed in Table I is illustrated in Fig. 3.

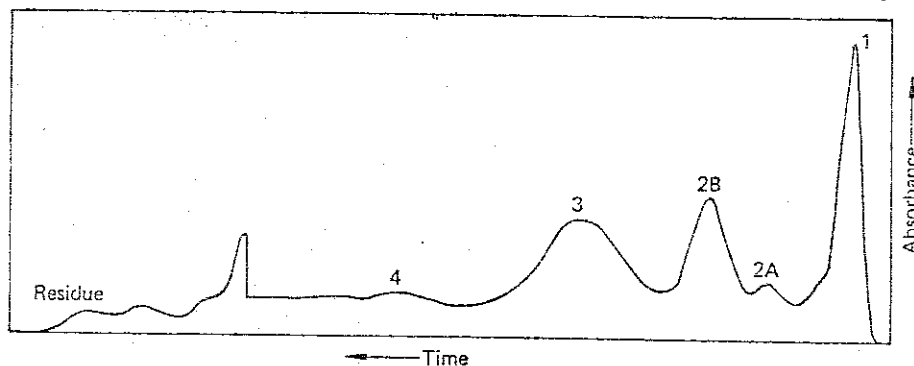

Fig. 3. Separation of the components of candicidin listed in Table I.

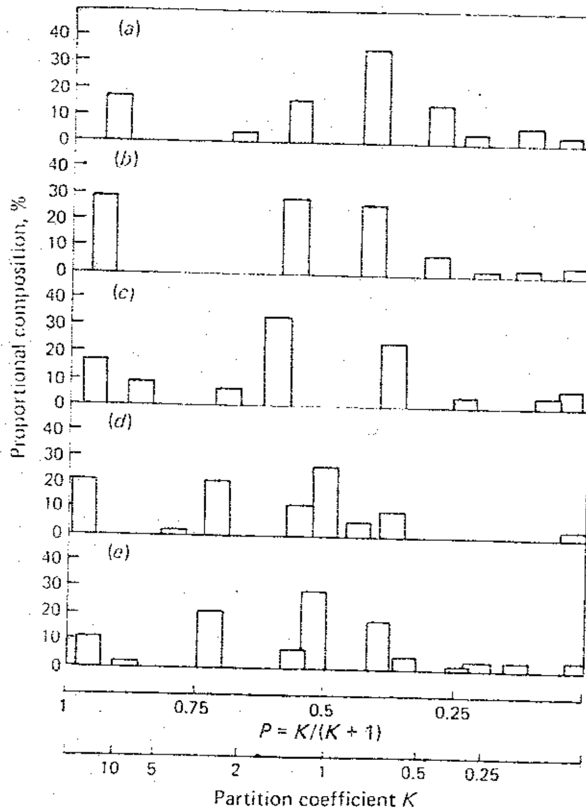

Fig. 4. Comparison of four polyene antibiotics using the coil planet centrifuge: (*a*), candicidin, PIRP; (*b*), levorin; (*c*), trichomycin; (*d*), hamycin sample 1; and (*e*), hamycin sample 2.

Fig. 4 compares diagrammatically similar data obtained for candicidin, trichomycin, levorin and hamycin. It shows clearly that the PIRP candicidin was different in nature and com-

TABLE I

COMPARATIVE COMPOSITIONS OF CANDICIDIN PREPARATIONS

$P = K/(K + 1)$ where K = partition coefficient.

Component*	PIRP		FDA working standard		Batches (7) from current production of two manufacturing sources			
	P	Content, %	P	Content, %	P		Content, %	
					Mean	Range	Mean	Range
1	0.91	17.5	0.93	15.4	0.96	0.91–1.00	20.0	8.8–29.2
2A	0.67	3.6	0.72	2.9	0.70	0.67–0.74	3.9	2.6–6.3
2B	0.55	16.0	0.56	14.6	0.56	0.53–0.59	17.7	13.9–23.2
3	0.41	36.1	0.40	28.8	0.40	0.38–0.42	31.4	23.6–44.5
4	0.29	14.4	0.28	20.4	0.28	0.27–0.30	14.4	7.9–21.1
Residue	0.22–0.04	12.7	0.19–0.02	17.9	0.23–0.02	0.25–0.01	12.6	7.9–19.4

* See Fig. 3.

position from trichomycin and hamycin. Two different batches of hamycin were available and differed markedly from one another. The sample of levorin resembled the candicidin much more closely, although the proportional composition was not included by the range of candicidin compositions noted; whether this sample of levorin is typical of present-day material could not be determined.

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Formation and Stability of Imidazole - Mercury Complexes with Some β-Lactam Antibiotics

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The standard method of the British Pharmacopoeia for the assay of β-lactam antibiotics is iodimetric titration. However, this method is subject to uncertain errors from iodine-absorbing impurities, so that for complex formulations it is normal to use microbiological assay. A chemical method for the determination of penicillins has been reported[1] that involves the use of imidazole and mercury(II) chloride to produce the stable mercury(II) mercaptides of their penicillenic acids (Fig. 1).

The imidazole - mercury assay has been applied by the BP Penicillins Panel, and formally proposed to the European Pharmacopoeia Commission, after a successfully concluded comparative study involving ampicillin, benzylpenicillin and phenoxymethylpenicillin. The general procedure is as follows.

Prepare a solution to contain 50 μg ml^{-1} of the penicillin or 200 μg ml^{-1} of the cephalosporin in water, and pipette 2 ml of this solution into each of two test-tubes labelled "test" and "blank." Dissolve 9.1 g of imidazole (recrystallised from toluene) in approximately 70 ml of water, adjust to the relevant pH shown in Table I using hydrochloric acid, and add water to 100 ml. Prepare the imidazole - mercury solution by adding dropwise, with continuous stirring, 1 ml of a 0.27% m/V solution of mercury(II) chloride in water to each 10 ml of imidazole stock solution taken. Pipette 10 ml of this solution into the tube marked "test." Prepare the imidazole working solution by adding 1 ml of water to each 10 ml of imidazole stock solution taken. Pipette 10 ml of this solution into the tube marked "blank." Stopper both tubes, mix, place in a water-bath at the temperature and for the time stated in Table I, then cool to room temperature and measure the absorption of the "test" solution at the appropriate wavelength using the "blank" solution as reference.