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Thin-layer chromatography on Sephadex for the identification of antibiotics

In Sephadex thin-layer chromatography a buffer solution is most commonly used instead of organic solvents. Due to the absence of organic solvents in the developed Sephadex chromatogram a direct modified bioautography technique could be applied, the advantages of which are high sensitivity and specificity[1]. This novel combination of Sephadex TLC and bioautography was applied to problematic qualitative analyses of mixtures of particular antibiotics. In this technique a close contact between the Sephadex chromatogram and an agar plate seeded with a suitable microorganism was maintained for 30 min. The R_F value of the antibiotic material was determined by the location of the area of inhibited growth on the agar plate after incubation. An advantage of the combination of Sephadex TLC and bioautography is the elimination of false inhibition zones, due to incomplete removal of organic solvents which sometimes occurs with conventional chromatographic methods.

Experimental

For descending chromatography a modified Determann sandwich arrangement[2] was used. The upper plate, made of Perspex, was fitted with rubber strips (height 2 mm) at the sides and with a sampling slit to be closed with tape. Contact with the upper and lower buffer reservoirs was established by means of wads of filter paper (Whatman No. 120).

A gel suspension was prepared by mixing 33 g Sephadex G-15 (40–120 μg) with 100 ml of 0.025 M phosphate buffer (KH_2PO_4–NaOH, pH 6.0) containing 0.5 M NaCl. After swelling for at least 30 min, the suspension was spread in an even layer of 0.5 mm thickness on thoroughly degreased 20 $\times$ 20 cm glass plates. The plates were allowed to dry at room temperature for about 1 h until the surface was no longer shiny and were transferred to a moist chamber where they were kept in a horizontal position for at least 24 h.

Before use the plates were mounted at a 30-degree inclination and were provided with the presoaked paper flaps and additional strips of filter paper (Whatman No. 120) to ensure good contact and closure of the chamber.

Suitable amounts of the sample in a volume not exceeding 2 μl were applied to the wet surface through the sample slit by means of Drummond's Microcap pipettes. Immobility of the mobile phase during application was assured by varying the humidity of the upper paper flap and was checked with a spot of Blue Dextran. The run, initiated by filling the upper reservoir, lasted 60–90 min.

After development the Sephadex chromatoplate was taken from the chamber and was pressed on a seeded agar plate covered with a sheet of lens tissue paper. After "printing" for 30 min, the chromatoplate was taken off, the lens tissue paper was removed and the agar plate was incubated at the optimal growth temperature of the test organism.

The agar plates used (20 $\times$ 20 cm) were prepared with 30 ml of the seeded agar medium; media compositions are given in Table I.

Results

Good separations were obtained (Fig. 1). The results with 17 antibiotics are presented in Table I. Traveling rates are expressed relative to that of penicillin-G, which is assigned the value of 1.0. This value of 1.0 is valid for the sodium, potassium and procaine salts.

TABLE I

SEPARATION OF ANTIBIOTICS BY SEPHADEX THIN-LAYER CHROMATOGRAPHY

Bioassay conditions: (A) *Bacillus subtilis* ATCC 6633; medium 4 (ref. 4) in 0.05 M KH_2PO_4–NaOH buffer pH 6.5 (ref. 5); incubation 18 h, 30°. (B) *Staphylococcus aureus* ATCC 6538 P; medium 1 (ref. 4) in 0.1 M triethanolamine–HCl buffer pH 8.0 (ref. 5); incubation 18 h, 30°. (C) *Bacillus subtilis* ATCC 6633; medium 1 (ref. 4) in 0.1 M triethanolamine–HCl buffer pH 8.0 (ref. 5); incubation 18 h 30°. (D) *Saccharomyces cerevisiae* ATCC 9763; medium ref. 6; incubation 18 h 30°.

Antibiotic	*Relative travelling rate*	*Amount assayed (µg)*			
		A	*B*	*C*	*D*
Penicillin-G	1.0	0.1	0.4	1.0	
Penicillin-V	0.8	0.1			
Propicillin (L and D)	0.9	0.1			
Nafcillin	0.5	0.1			
Ampicillin	1.4	0.1			
Dicloxacillin	0.5	0.2			
Tetracycline	0.7	0.05			
Oxytetracycline	0.7	0.05			
Chlortetracycline	0.6	0.05			
Neomycin	1.8		1.0		
Kanamycin	2.0		2.0		
Staphylomycin	0.6[a]	2.0	0.5		
Streptomycin	1.7	2.0	2.0	0.2	
Oleandomycin	1.7	0.5	0.5	0.5	
Pimaricin	0.7				0.2
Nystatin	0.2				1.0
Amphotericin-B	0.2				1.0

[a] Tailing.

The amount of antibiotic demonstrated depends on the bioassay conditions; variation in the test organisms permits a further differentiation of antibiotics on the base of the antibiotic spectrum.

As expected[3], the resolution on Sephadex G-15 depends on adsorption rather

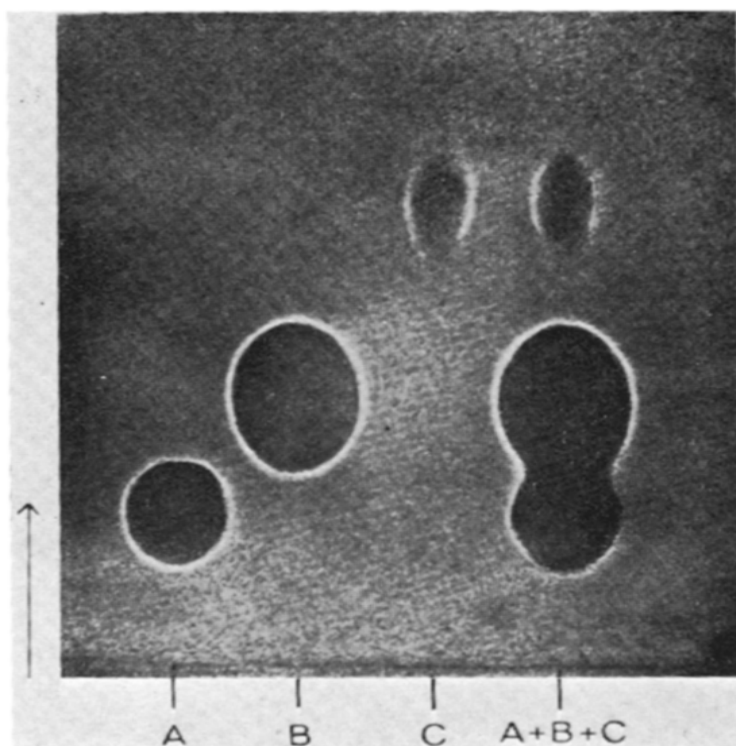

Fig. 1. Sephadex thin-layer chromatogram of three antibiotics, visualized by bioautography. (A) 0.2 μg dicloxacillin, sodium salt; (B) 0.1 μg penicillin-G, procaine salt; (C) 2 μg streptomycin sulphate. Bioassay condition A (Table I).

than on graded accessibility. This is demonstrated by a comparison of the travelling rates of staphylomycin (mol. wt. = 555–590) and streptomycin (mol. wt. = 581).

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