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Note

Paper chromatography of tobramycin and some related compounds

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Tobramycin is an antibiotic produced in submerged fermentation by *Streptomyces tenebrarius* (ATCC 17920). This antibiotic, previously described as tenebrimycin and tenemycin[1], is an aminoglycoside whose structure has been reported in detail by Koch and Rhoades[2]. Several reports in the literature include paper chromatography of tobramycin[1,3]. The best of the reported methods uses a system[4] of water-saturated *n*-butyl alcohol containing 2% *p*-toluenesulfonic acid; however, this system requires a 40-h development time and does not satisfactorily separate factor 5 from 6, especially when the ratio of 5 to 6 is small.

This paper reports a new chromatographic system which requires only 6 h of development time. This method, when combined with the differential organism, *Sarcina lutea*, can detect 1% of factor 5 in factor 6. The system has been in use for over three years with few problems.

EXPERIMENTAL

Chemicals

All chemicals were reagent grade and were used without further purification. The materials for the chromatography were supplied by Dr. W. M. Stark, also of Eli Lilly and Company. Structures for tobramycin and its related compounds are shown in Fig. 1.

Solvent systems

Acetone was mixed 55:45 with deionized water and used for the developing solvent. The paper was developed in a descending manner in a chromatographic chamber 24 in. tall and 12 in. in diameter. The tank was filled with 1 l of acetone–deionized water (85:15) for purposes of equilibration. The tank was allowed three days for equilibration, and remained stable for several months.

Detection systems

Ninhydrin. 0.3 g of ninhydrin was dissolved in 100 ml of ethanol and mixed with 3 ml of glacial acetic acid. The ninhydrin solution is sprayed upon the chromatogram, and the chromatogram heated at 100° for 15 min.

B. subtilis. One millilitre of *Bacillus subtilis*, ATCC 6633, light transmission 25%, was added to mycin agar buffered at pH 8.5.

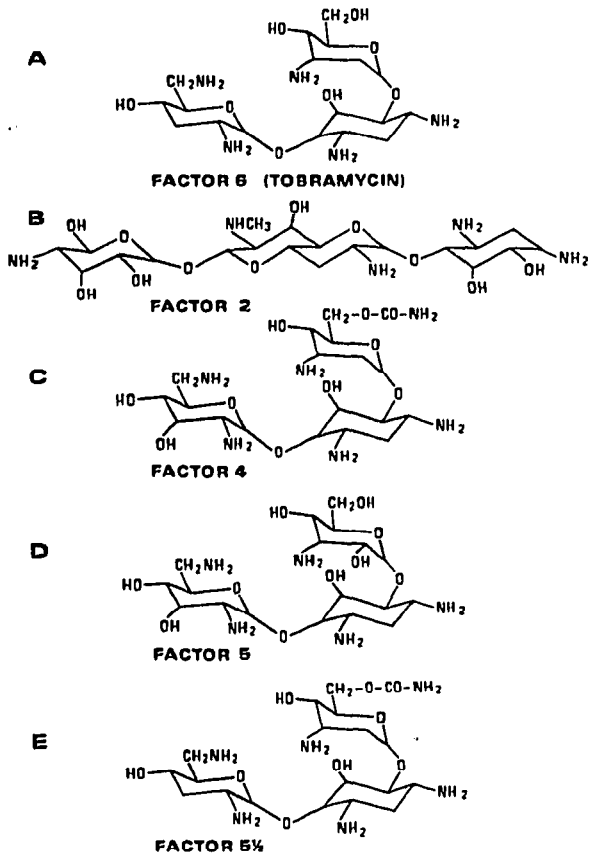

Fig. 1. Structures of tobramycin and four related compounds.

S. lutea. Three millilitres of *Sarcina lutea*, ATCC 9341, stock solution were added to penicillin seed agar buffered at pH 6.7. The stock solution was prepared so that when diluted 1:100, it produced a 25% light transmission at 530 nm.

Mycin agar. 1.5 g of beef extract, 6.0 g of yeast extract, 6.0 g of peptone, and 17.5 g of agar were dissolved in purified water to make 1000 ml. All ingredients were dissolved with heat and stirring, and the pH of the final solution was brought to pH 8.5.

Penicillin seed agar. 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar were mixed and prepared in purified water to make 1000 ml. The pH was adjusted to 6.7.

Buffer for chromatography paper. 159 g of tripotassium phosphate was dissolved in approximately 1 liter of deionized water. The pH was adjusted to 10.0 with concentrated phosphoric acid, and the volume adjusted to 1 liter.

Equipment

Whatman No. 1 chromatography paper was used throughout, and was obtained from H. Reeve Angel (Clifton, N.J., U.S.A.). Micropipets (Microcaps®) from Drummond (Broomall, Pa., U.S.A.) were used for making sample applications. Disposable bioautographic dishes were obtained from Lancaster Glass (Lancaster, Ohio, U.S.A.).

Procedure

The paper chromatographic chamber was prepared with the equilibration solvent, and allowed to equilibrate for three days before use. Whatman No. 1 paper cut into 7½ in. × 18 in. sheets was dipped and pulled through the buffer. The sheets were allowed to dry for three days before use. Three sub-procedures were then employed, depending upon the detection system. Solutions were prepared at 1 mg/ml in water for *B. subtilis* detection, at 40 mg/ml for *S. lutea* detection, and at 10 mg/ml for ninhydrin detection. In all cases, 2 μl of the solution was spotted onto the paper chromatogram using a micropipette. The chromatogram was then developed with the developing solvent for approximately 6 h, or until the solvent front had reached 1 in. from the bottom of the chromatogram. The paper chromatogram was then removed from the developing chamber. The chromatogram then was handled in two ways, depending upon the sub-procedure. For micro-organism detection, the chromatogram was merely fanned four to five times in the air, and applied onto a gelled sheet of the appropriate seeded agar. For the ninhydrin detection system, the developed paper was hung in a fume hood until the paper was dry (approximately 2 h).

RESULTS AND DISCUSSION

Fig. 2 shows the separation and the relative sensitivity of *B. subtilis* and *S. lutea* for tobramycin and the other metabolites of *Streptomyces tenebrarius*. The detection

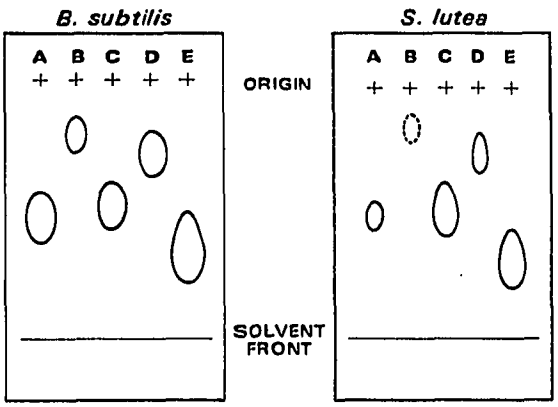

Fig. 2. Bioautographic chromatograms of tobramycin and four related compounds. Two micrograms of each compound were spotted on both tapes. The lanes correspond to the compounds A–E of Fig. 1.

with ninhydrin is not illustrated since the densities of the spots for all compounds were equal.

As can be seen in the second chromatogram, *S. lutea* has very little sensitivity for tobramycin, but still retains sensitivity for factor 5. This sensitivity differential allows large loadings of factor 6 without attendant overlap onto factor 5. Fig. 3 shows a chromatogram where 1 % of factor 5 has been added (spiked) into tobramycin. Factor 4 could remain a serious interferent except that it is deliberately hydrolyzed to factor 5 during manufacture of tobramycin and even if present, it can be detected in low level by the IR absorption at 5.8 μ.

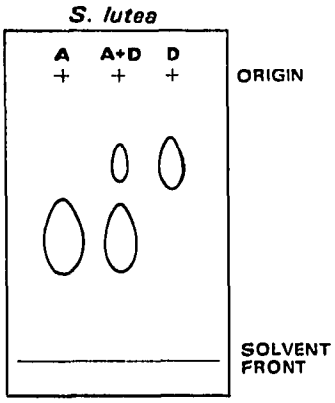

Fig. 3. Bioautographic chromatogram of tobramycin: 1% factor 5 in nebramycin, and a 5% equivalent of factor 5 alone. 80 μg of nebramycin is spotted. The compounds on the lane follow the letter designation of Fig. 1.

TABLE I

SOLVENT SYSTEMS FOR PAPER CHROMATOGRAPHY AND $R_F \times 100$ VALUES ON BUFFERED PAPER IN VARIOUS SOLVENT SYSTEMS

Solvent system	*Development time (h)*	$R_F \times 100$ *value*				
		Factor 6	*Factor 2*	*Factor 4*	*Factor 5*	*Factor 5½*
Ethyl acetate–ethanol–water (25:20:6)	3.5	65	47	57	57	65
Acetone–ethanol–water (4:1:1)	4.5	65	48	57	58	65
Acetone–water (3:1)	8	16	10	16	10	27
Acetone–water (55:45)	6	56	27	54	34	76

Several other solvent systems were investigated before choosing the chromatographic system described here. In Table I, $R_F \times 100$ values are given for four other solvent systems. In all these systems the equilibrating solvent is the same as the developing solvent, and the paper is buffered at pH 10.0. It was found in the course of this investigation that for systems high in water, no resolution loss occurred if the water content of the equilibration solvent was reduced. The choice of an 85% acetone in water equilibration solvent was made on an arbitrary basis (it being convenient for another chromatographic assay).

Thin-layer (TLC) systems for tobramycin on silica, cellulose, and polyamide were also tried. It was felt by this author that all TLC systems tended to streak more than the paper systems. Some attempts were made to buffer the TLC absorbents, so as to minimize streaking, but paper in all cases gave better results.

REFERENCES

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