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AMINOGLYCOSIDE ANTIBIOTICS: THIN-LAYER CHROMATOGRAPHY, BIOAUTOGRAPHIC DETECTION AND QUANTITATIVE ASSAY

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SUMMARY

A sensitive method is described for the identification of aminoglycoside antibiotics, for the detection of agents exhibiting biological activity in the course of fermentation, isolation and purification, and for following their chemical conversion. In the multi-component nebramycin complex the ratio of the factors can be established by the quantitative assay described.

INTRODUCTION

The separation and identification of biologically active compounds, e.g., antibiotics, are of increasing importance, and high-resolution thin-layer chromatography (TLC) is a simple, quick and selective method. In antibiotic research, microbiological detection is also of great importance, and different techniques are known for ensuring the diffusion of the test material into the culture medium layer containing agar and inoculated with the test organism, *e.g.*, (a) by spraying the warm, liquid, inoculated medium onto the chromatogram[1–4] (b) by tightly pressing the agar layer (previously prepared) on alumina[5] or glass plates[6,7] used directly for the chromatogram, (c) by inserting wet filter-paper between the chromatogram and the agar layer[8–10] and (d) by forming a collodion film from the chromatogram and placing it on the agar plate inoculated with the test organism[11]. Layers containing glass-fibre[12] and other layers[13,14] that exhibit significantly low adsorption towards the investigated substances are also employed.

To improve the detection of growing or fully developed microorganisms, specific dyes are used[1,2,15].

The separation of aminoglycoside antibiotics[16,17], especially of the individual factors of an antibiotic complex, is very difficult because they have only slight structural differences. The method described in this paper for the chromatography and bioautographic detection of different aminoglycoside antibiotics and components of the nebramycin complex[18] is suitable for the qualitative control of the enriched material and also for following their chromatographic resolution and purification. The advantage of this method is that it can be used for both qualitative and quantitative control of the ratio of the different components during the course of fermentation, directly from the fermentation broth.

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EXPERIMENTAL

Materials and methods

All reagents and solvent were of analytical-reagent grade.

Thin-layer plates (20 × 20 cm) were prepared from silica gel G (according to Stahl; Reanal, Budapest, Hungary). Also, 20 × 2 × 0.3 and 16 × 2 × 0.3 cm glass plates were used for framing the chromatograms after development in Desaga glass chambers. Standard solutions for spotting were prepared by dissolving each substance in water (100 μg/ml) (with the nebramycin complex the concentration of the individual components 2, 4 and 5′ should not be less than 20%).

The two developing solvents employed were (A) methyl ethyl ketone (MEK) –96% ethanol–25% ammonia solution (1:1:1) and (B) chloroform–methanol–25% ammonia solution (1:7:4). Both solvent systems were mixed freshly before use.

For staining the bioautograms, 0.5-1% tetrazolium blue (TB) (2,2′,5,5′-tetraphenyl-3,3′-dimethoxy-4,4′-biphenyleneditetrazolium chloride) (Reanal) solution and 0.02% tetrazolium violet (INT) [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride] solution (Fluka, Buchs, Switzerland) were employed.

The microbiological assay was prepared with *Bacillus subtilis* ATCC 6633 as test organism inoculated into the culture medium, containing 1 l of bouillon (1 kg of minced beafheart, boiled for 2 h with 2 l of tap water and subsequently filtered), 5 g of peptone and 14 g of agar-agar (fibrous). The pH was adjusted to 8.4 with sodium hydroxide solution before sterilization.

Procedure

Silica gel plates with a 0.25-mm thick layer were prepared with a Camag or Desaga TLC plate coater, dried at room temperature for 1–2 days and used without further pre-treatment. Leaving a 2.5-cm frame, lines were drawn before use to ensure separate 1.5-cm wide tracks.

The amount of sample to be applied was chosen according to the properties of the substance being investigated. For nebramycin a solution containing 0.1–0.5 μg of test substance was spotted with a micropipette. Occasionally a larger amount, 1–5 μg, of the substance must be spotted and chromatographed when the aim of the analysis is the detection of impurities or the proving of purity. On each plate a standard solution was also spotted on the track in addition to the sample. For the separation of the nebramycin complex and of the different aminoglycoside antibiotics solvent systems A and B, respectively, were applied. The plates were developed for a distance of 15 cm at room temperature and air-dried.

For the microbiological detection the agar medium was melted, then cooled to 50°C and 1/10th volume of an inoculum of the test organism was added (viable count $0.9 \cdot 10^7$). A 25-ml volume of this medium, inoculated with *Bacillus subtilis*, was cautiously poured on to glass-framed plates (fixed by adhesive tape) in order to ensure a cover of uniform thickness on the silica gel surface. Then it was covered with a 20 × 20 cm glass chromatographic plate and incubated for 10–16 h at 37°C.

Quantitative assay was performed by using a calibration graph (for a good evaluation, identical volumes of solutions with different concentrations from the standard and test solution must be spotted).

RESULTS AND DISCUSSION

Figs. 1 and 2 demonstrate that the major nebramycin components show readily discernible, completely resolved inhibition spots, even if less than 0.5 μg was applied. This assay can also be used directly for fermentation broths. By measuring samples obtained at the required intervals, the formation of individual components and their approximate ratios can be followed. For more exact assays, identical amounts from different concentrations of the same sample are spotted, which permits also the detection of minor factors.

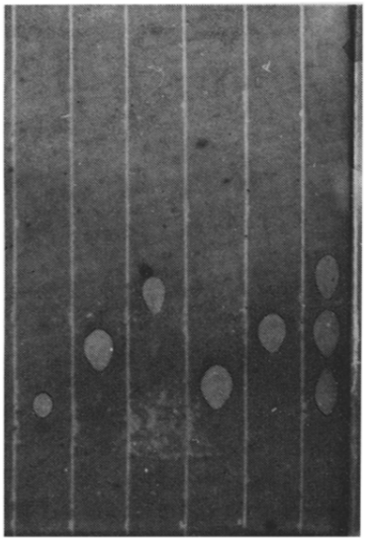
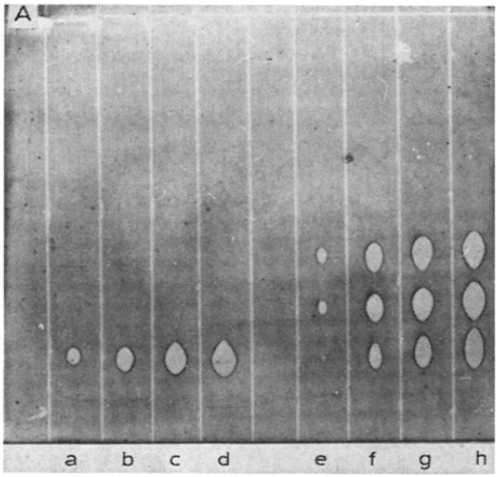


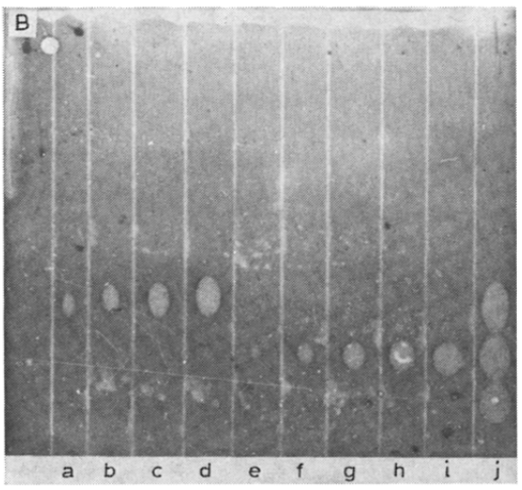
Fig. 1. Bioautogram of a thin-layer chromatogram of nebramycin components. Left to right: nebramycin 2, 4, 5′, 5, 6, complex. Developing solvent: A. Test organism: *Bacillus subtilis*. Detection: INT.

In Fig. 3, a bioautogram prepared for the determination of apramycin and carbamoyl-tobramycin formed in the fermentation broth, and of tobramycin transformed by basic hydrolysis, is illustrated.

For quantitative assays a calibration graph is constructed from the inhibition spots of standard materials. The areas of sample spots applied to the same plate are measured and compared with the corresponding values on the calibration graph. A graph of area of the inhibition spots against the logarithm of the concentration of the substances applied gives a straight line; 0.1–0.3 μg amounts are suitable for constructing the calibration graph. Individual calibration graphs have to be prepared for each component because of their different specific activities. Uniform thickness of the silica gel and agar layers is essential.

The method described is suitable for the determination of the components of the nebramycin complex, as their desorption from the thin-layer plate (silica gel G) is sufficient for quantitative assay.





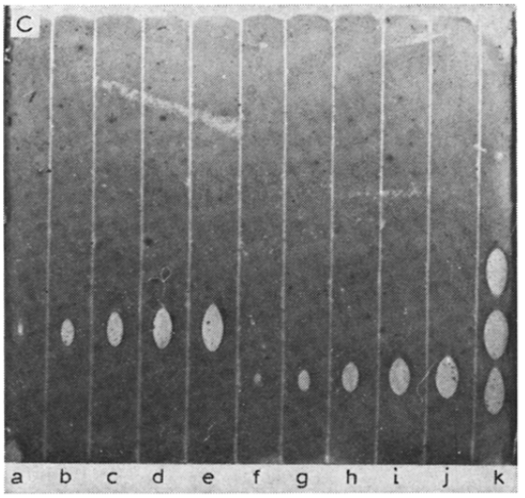


Fig. 2. (A) Bioautogram of various amounts of nebramycin components. Experimental conditions as in Fig. 1. (a)–(d) Nebramycin 2 (apramycin): 0.2, 0.3, 0.4, 0.5 μg; (e)–(h) nebramycin complex with the following concentrations of 2, 4 and 5′: (e) 0.1, 0.05, 0.05 μg; (f) 0.2, 0.1, 0.1 μg; (g) 0.3, 0.2, 0.2 μg; (h): 0.4, 0.3, 0.3 μg. (B) (a)–(d) nebramycin 5′ (carbamoyl-tobramycin): 0.05, 0.1, 0.2, 0.3 μg; (e)–(i) nebramycin 4 (carbamoyl-kanamycin B): 0.05, 0.1, 0.2, 0.3, 0.4 μg; (j) nebramycin complex. (C) (a)–(e) nebramycin 6 (tobramycin): 0.05, 0.1, 0.2, 0.25, 0.3 μg; (f)–(j) nebramycin 5 (kanamycin B): 0.05, 0.1, 0.2, 0.25, 0.3 μg; (k) nebramycin complex.

Fig. 4 shows a chromatogram developed with solvent B. The method can be applied to the separation, detection and identification of various aminoglycoside antibiotics. The R_F values of substances in solvent B are given in Table I.

For the measurement of the specific activities of individual substances the corresponding microorganism is inoculated into the medium and poured on to the plate. In addition to *Bacillus subtilis*, *Sarcina lutea* (Hussey) and *Mycobacterium phlei* were also applied.

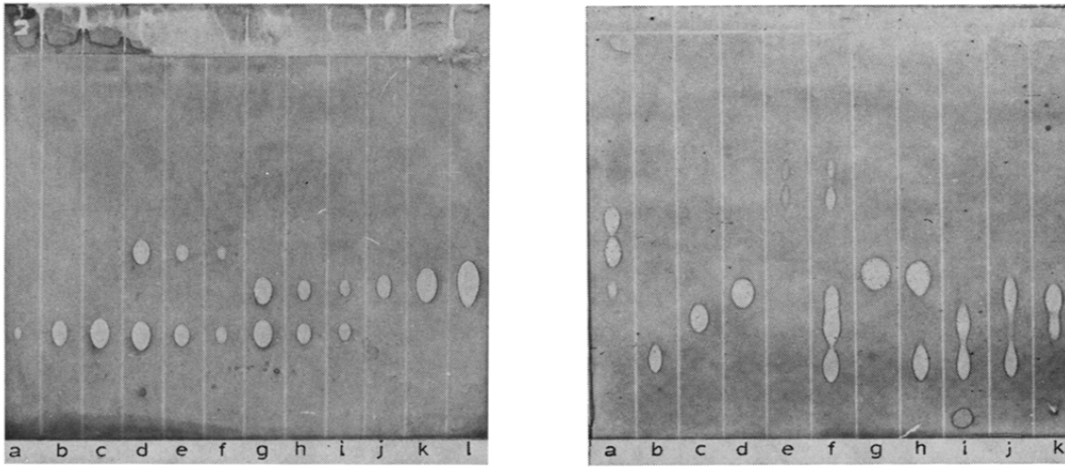


Fig. 3. Bioautogram of a fermentation broth in the original state and after basic hydrolysis (semi-quantitative determination). Experimental conditions as in Fig. 1. (a)–(c) Apramycin in different concentrations; (d)–(f) fermentation broth, serial dilution; (g)–(i) fermentation broth, after hydrolysis, serial dilution; (j)–(l) tobramycin, in different concentrations.

Fig. 4. Thin-layer chromatogram of some aminoglycoside antibiotics. Developing solvent: B. Detection: bioautography. Test organism: *Bacillus subtilis*. (a) Nebramycin complex, 0.4 μg; (b) neomycin (N), 0.1 μg; (c) paromomycin (P), 0.2 μg; (d) kanamycin (K), 0.3 μg; (e) gentamycin (G), 0.3 μg; (f) N + P + K + G, 0.15 + 0.1 + 0.1 + 0.3 μg; (g) neamine, 0.5 μg; (h) N + neamine, 0.15 + 0.5 μg; (i) N + P, 0.1 + 0.1 μg; (j) N + K, 0.1 + 0.1 μg; (k) P + K, 0.1 + 0.1 μg.

TABLE I

R_F VALUES OF AMINOGLYCOSIDE ANTIBIOTICS IN SOLVENT SYSTEM B

Compound	$R_F \times 100$
Neomycin	12
Paromomycin	23
Kanamycin A	30
Nebramycin (2, 5; 4, 6; 5′)	32; 42; 51
Gentamicin (C_{1a}; C_1, C_2)	56; 63
Sisomicin	60
Neamine	37
Amikacin	6

Investigations can be carried out with clone colonies of unidentified micro-organisms producing active compounds, grown on a solid medium. A small disc of the single colony is cut out and placed on the silica gel chromatographic plate for 5–15 min. The biologically active substances will diffuse into the silica gel and then the investigation can be performed following the method described.

The visibility of the inhibition zones on the bioautogram can be improved by spreading a 0.5–1.0% TB solution on the plate after incubation for 12–16 h. Approximately 60 min later the rim of the spots becomes pronounced. Staining the plates with a solution of INT gives colourless spots on a deep red or wine-coloured back-

ground. The colour becomes most intense within 5-10 min, and good photographs can be taken.

The detection limits are as follows: nebramycin components 4, 5, 5′ and 6, 0.05 μg; nebramycin component 2, 0.1 μg; gentamicin, 0.2 μg; and neomycin, paromomycin and kanamycin, 0.1 μg. The sensitivity can be increased by applying thinner silica gel layers and, especially, thinner agar layers or more sensitive micro-organisms.

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