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SEPARATION OF NEBRAMYCIN COMPONENTS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A thin-layer chromatographic method has been developed for the detection of major nebramycin components, for the separation of tobramycin from other components and for studying the hydrolysis of carbamoyl derivatives and procedures for isolation and purification.

A sensitive method has also been established for the detection of kanamycin B in tobramycin and for the assay of apramycin in kanamycin B.

INTRODUCTION

The nebramycin complex, which is a mixture of aminoglycoside-type antibiotics, is produced by a strain of *Streptomyces tenebrarius*[1,2]. According to studies by Stark *et al.*[3], three major components are formed by the microorganism, namely apramycin (factor 2), carbamoyl kanamycin B (factor 4) and carbamoyl tobramycin (factor 5'). Components 1, 1' and 3, described by Thompson and Presti[4], are produced only in negligible amounts, whereas 5 (identical with kanamycin B) and 6 (tobramycin) can be prepared by the hydrolytic cleavage of 4 and 5, respectively[5].

According to the literature, their detection is carried out by paper chromatography, although this method results in poor separations, even if continued for 40 h. In an improved procedure[6], component 5 (present as an impurity) can be detected in tobramycin (component 6) after a run of 6 h, although the spots are unsatisfactorily separated if factors 2, 4 and 5' are additionally present. By means of thin-layer chromatography elongated, poorly resolved spots were obtained[5,6].

By means of the thin-layer chromatographic method described here, the major components of nebramycin could be separated satisfactorily within 2 h with a run of about 15 cm or, by using high-performance thin-layer chromatography (HPTLC) plates in 10 min with a run of 2.5–5 cm. In addition, the hydrolytic cleavage of the components (4 to 5 and 5' to 6) could also be followed.

EXPERIMENTAL

Materials and apparatus

Analytical-reagent grade chemicals were applied without further purification.

Thin-layer plates (0.25-mm layer) were prepared from silica gel G for chromatography according to Stahl. High-performance thin-layer plates (E. Merck, Darmstadt, G.F.R.) were used for linear and circular chromatography (HPTLC).

The developing solvent was methyl ethyl ketone–ethanol (96%)–25% ammonia solution (1:1:1). Detection of spots was effected with the following reagents: (A) ninhydrin solution, 0.5% in acetone; (B) vanillin (1.7 g) + distilled water (30 ml) + 96% ethanol (40 ml) + orthophosphoric acid (100 ml); (C) 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride; Pierce, Rockford, Ill., U.S.A.), 0.25 mg/ml in methanol.

A Desaga chromatographic separating chamber and a Camag U-chamber for circular development of HPTLC plates were used.

Procedure

A solution of the nebramycin complex (or a mixture of the components) and a test solution containing 20–200 or 0.2–20 μg of each substance were spotted on thin-layer plates (dried at room temperature, inactivated and divided by lines to ensure separate tracks) or on HPTLC plates. The chromatogram was developed at room temperature in the solvent system described above (length of run about 15 or 5 cm on HPTLC plates or 2.5 cm in circular chromatography). The plates were dried and developed with ninhydrin by spraying the layer thoroughly. The spot could be detected at room temperature after about 30 min (or after 10 min at 100°).

For detection with vanillin, the plates were placed in an oven of 120° for 20 min following spraying with the reagent. The spots can also be detected with other reagents too, namely phloroglucinol–sulphuric acid[7], chlorine–tolidine (or iodine vapour) or, in specific cases, thymol–sulphuric acid[8].

For the detection of small amounts of compounds, NBD chloride reagent was used. The sprayed plate was heated in an oven at 120° for 10 min, cooled to room temperature and then re-chromatographed in methanol or absolute ethanol (in the same direction as in the first run) to wash out the excess of the reagent. Fluorescent spots were detected under a Universal UV lamp (Camag TL 900) at 366 nm.

RESULTS AND DISCUSSION

The method is suitable for the assay of eluates obtained by ion-exchange chromatography in the course of the isolation of nebramycin from fermentation broth, the study of fractions formed during chromatographic separation and the control of purification procedure.

Fig. 1 shows the chromatogram of a nebramycin complex, containing mainly components 2, 4 and 5′. Fig. 2 shows the chromatogram of individual nebramycin components. The R_F values of the individual components are given in Table I.

Table I and Figs. 1 and 2 demonstrate that components 2 and 5, and also 5′ and 6, cannot be separated on silica gel in this solvent system (it should be mentioned that in the course of fermentation neither component 5 nor 6 is formed). As only tobramy-

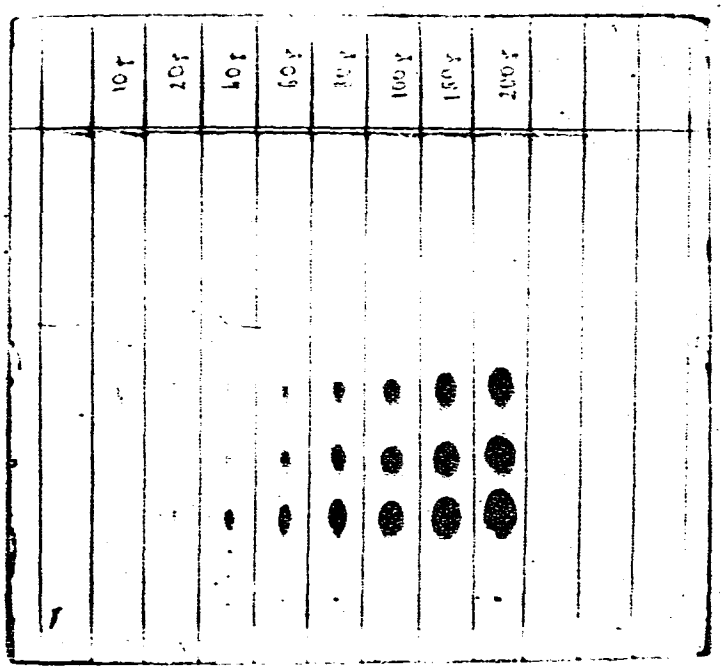

Fig. 1. Silica gel G thin-layer chromatogram of the nebramycin complex (10, 20, 40, 60, 80, 100, 150, 200 μg). Detection with ninhydrin.

cin (component 6) is used as a drug, in the course of isolation component 4 is transformed into 5, and 5′ is transformed into 6 by hydrolytic cleavage and subsequent chromatography. Their conversion (hydrolytic cleavage of the carbamoyl group) might be followed.

The proposed method is also applicable to the detection of minor components, especially in the enriched fractions obtained in the course of chromatographic separation. In this instance the plates are run twice, with drying in between.

The nebramycin components can be detected with ninhydrin, chlorine–tolidine or phloroglucinol–sulphuric acid (sensitivity 2–5 μg).

Detection with vanillin results in spots with a variety of colours and intensities.

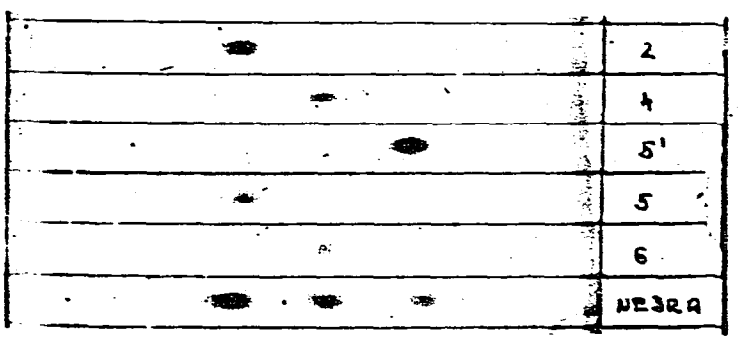

Fig. 2. Silica gel G thin-layer chromatogram of major nebramycin components (nebramycin 2, 4, 5′, 6, complex). Detection with ninhydrin.

TABLE I

R_F VALUES OF INDIVIDUAL COMPONENTS OF NEBRAMYCIN

Component	R_F
Nebramycin 2 (apramycin)	0.31
Nebramycin 4 (carbamoylkanamycin B)	0.49
Nebramycin 5 (kanamycin B)	0.36
Nebramycin 5′ (carbamoyltobramycin)	0.66
Nebramycin 6 (tobramycin)	0.52

Nebramycin component 2 is the most sensitive and gives a brown colour. The colours of the other components are different from this, a variety of green-grey shades appearing and exhibiting light blue fluorescence under UV light. The variety of colours shown by the different minor components and also their different intensities may facilitate the development of an improved procedure for the isolation, purification and identification of the individual components. The higher colour intensity of nebramycin component 2 may be utilized for its detection in nebramycin component 5. The brown spot characteristic of nebramycin component 2 is visible at concentrations down to 0.2–0.5 μg, whereas the other components can be detected only in at concentrations higher than 5–10 μg, or revealed under UV light in 1–2-μg amounts.

Thymol–sulphuric acid reagent, similarly to detection with vanillin, gives the greatest sensitivity with nebramycin component 2 (1–2 μg can be detected). All of the other components exhibit only pale spots, even at concentrations of 10–20 μg, although their visibility can be improved by using UV light at 366 nm. Consequently, this procedure is suitable for the specific detection of nebramycin component 2 in kanamycin B or in a mixture of other substances.

As fluorescence is 10–100 times more sensitive than colorimetric procedures, fluorigenic labelling techniques have also been investigated. 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD chloride) was applied to the detection of small amounts of the compounds, reacting with primary and secondary amines to yield non-fluuorescent hydrolysis products. NBD chloride has been described as a sensitive fluorogenic reagent for amines and amino acids by Ghosh and Whitehouse[9], and for other amine-containing compounds by Benjamin *et al.*[10] and Kabasakalian *et al.*[11].

In our experiments, the derivatives were prepared from the separated substances on the previously developed chromatogram by spraying the plates with the reagent and heating them to the appropriate temperature. The derivatives of nebramycin components appeared as yellow fluorescent spots. Re-chromatography in methanol (or absolute ethanol) resulted in an increase in sensitivity. The limit of the detection is 0.1–0.2 μg.

Detection of substances is also possible on chromatograms prepared from appropriately diluted (filtered) fermentation broth.

By comparing visually the intensities of the fluorescent spots of unknown samples with those of standard solutions, the approximate concentrations of the

former could be determined. This method is suitable for the determination of the purity of the compounds.

According to our observations, the shape of the spot when an aminoglycoside antibiotic is applied in the form of the base differs from that of the corresponding sulphate salt, probably owing to their different adsorption on the silica gel.

The pre-coated plates prepared for HPTLC are suitable for rapid analyses. The compounds separate well both on linear and circular chromatograms (Camag U-chamber). For detection the methods described above, mainly with chlorine–tolidine or NBD chloride reagent, were used.

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