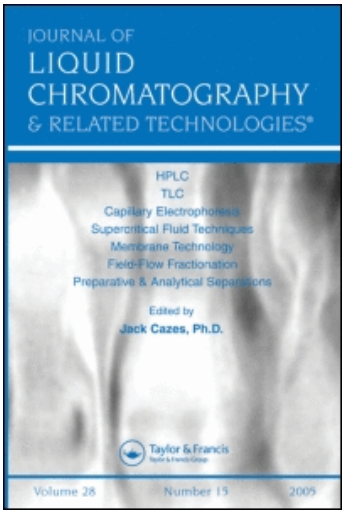

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Determination of the Major Factors of Fermentation of the Nebramycin Complex by High Performance Liquid Chromatography

János Harangi[a]; Magdolna Deák[a]; Pál Nánási[a]; György Bacsa[b]
[a] Institute of Biochemistry, L, Kossuth University, Debrecen, Hungary [b] BIOGAL Pharmaceutical Works, Debrecen, Hungary

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DETERMINATION OF THE MAJOR FACTORS OF FERMENTATION OF THE NEBRAMYCIN COMPLEX BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

János Harangi, Magdolna Deák, Pál Nánási

Institute of Biochemistry, L. Kossuth University
H-4010 Debrecen, Hungary

and

György Bacsa

BIOGAL Pharmaceutical Works
H-4042 Debrecen, Hungary

ABSTRACT

For the determination of the major factors (tobramycin, kanamycin B, apramycin) from the fermentation broth a new method has been developed with combination of some earlier published method. In this method the protein content of the mixture was removed by treatment with tris-(hydroxymethyl)-aminomethane followed by centrifuging then the antibiotic content was derivatized by 1-fluoro--2,4-dinitro-benzene. The mixture was analysed on a reversed phase column.

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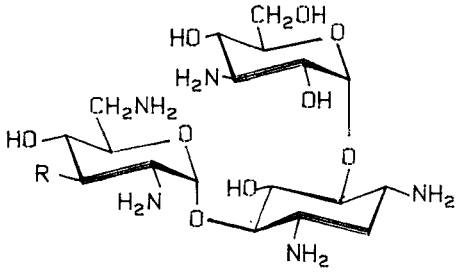
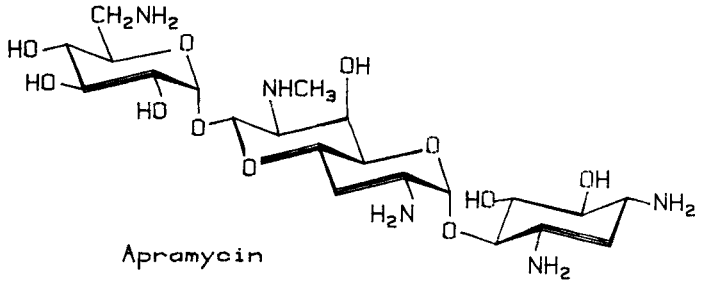
INTRODUCTION

The antibiotics tobramycin, kanamycin B and apramycin (Fig. 1), producing upon fermentation of the nebramycin complex, possess wide spectrum of activity and relatively low toxicity, providing favourable utilization in veterinary, and indeed, in human therapy. The control and qualification of the products obtained on fermentation are essential requirements of the application of such antibiotic substances.



Kanamycin B: R=OH
Tobramycin: R=H



Apramycin

Figure 1. Structures of the antibiotics

Several attempts have been made so far for the analysis of aminoglycoside-type antibiotics, involving a gas chromatographic method (1). One of the most severe difficulties of the high performance liquid chromatographic analysis of the aminoglycoside antibiotics is the detection. As these compounds do not have absorption in the ultraviolet range, sufficient chromatographic detection can be achieved only after derivatization. The prsence of amino groups in the molecule of these antibiotics allows the ion-exchange chromatographic separation and the detection can be performed either by refractive index detector or by fluorescence technique (2) preceiding derivatization of the separated components. For the separation and detection of the neomycin components Tsuji et al. (3) elaborated a method involving the reaction of the amino functions with 1-fluoro--2,4-dinitrobenzene (DNFB) and separation of the resulting N-dinitrophenyl derivatives on silicagel stationary phase using a chloroform-tetrahydrofurane-water system as the eluent. Although the separation was excellent, due to the large retention time of 2,4-dinitrophenol - the by-product of the derivatization reaction, formed from DNFB - the analysis required too long time. Barents at al. (4-6) and Elrod et al. (7) have reported on the analysis and determination of several aminoglycoside type antibiotics, also in serum, by the application of reversed phase column and derivatization with DNFB. For the detection and determination of several amino-

glycoside antibiotics a thin layer chromatographic method has been also reported recently (8).

Based on this background we attempted the separation and determination of the components formed upon nebramycin fermentation. First the isolated product mixture was investigated and then the method was extended to the examination of the fermentation broth, as well, assuring the fast and reliable control of the antibiotic level.

RESULTS AND DISCUSSION

Since there is no significant difference in the structure of the antibiotics to be separated (especially in the case of tobramycin and kanamycin B) the separation of the components of the mixture seemed to be difficult. No sufficient separation could be achieved using either ion exchange or ion-pair chromatographic technique (p-toluene sulfonic acid or pentane sulfonic acid ion pair forming agents, reversed phase column). In these cases the detection was very difficult and due to the low sensitivity of the refractive index detector the column was presumably overloaded.

The first successful experiments could be accomplished by using silicagel column and an eluent system similar to those reported in ref. 2, and these trials resulted in the sufficient separation of the three major components of the nebramycin complex (Fig. 2):

	k'	RT (min)
apramycin	3.64	6.40
kanamycin B	2.12	4.30
tobramycin	2.64	5.02

The retention time of the earlier mentioned by-product, 2,4-dinitrophenol, was 48 minutes, whereas the retention time of the last eluted component of the antibiotic mixture was below 10 minutes. Thus most part of the analysis involved the elution of 2,4-dinitrophenol making the

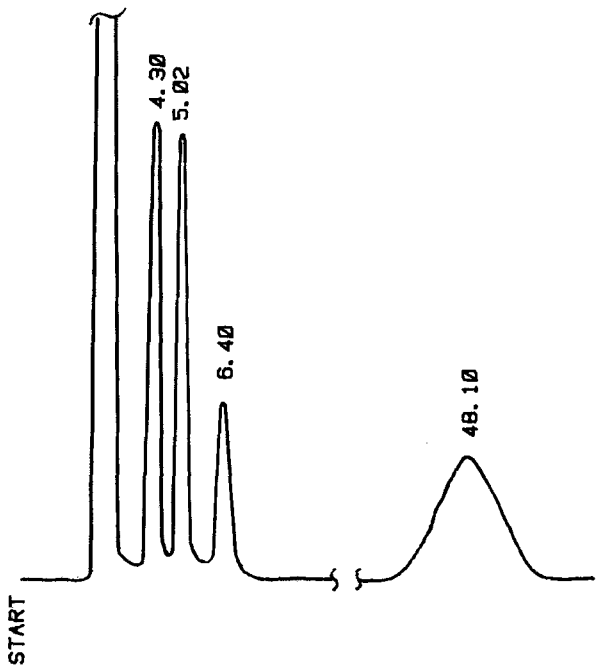


Figure 2. Separation of the major components of the nebramycin complex

method too long and tedious, therefore, another process was elaborated.

By the application of reversed phase technique and an eluent system (see Experimental) very similar to those reported for reversed phase column (4-7) satisfactory separation of the three antibiotics was obtained:

	k′	RT (min)
apramycin	6.97	9.56
kanamycin B	8.40	11.28
tobramycin	11.38	14.86

The excess of the derivatizing agent and the by-product of the derivatization reaction eluted at the outset of the chromatogram, so these materials did not disturb the determination. Using this procedure the three main components of the nebramycin complex could be simultaneously determined (Fig. 3).

The analysis of the antibiotics in the fermentation broth requires filtered and protein-free solution, as the presence of proteins and amino acides may disturb the determination process. In industry the removal of proteins from the fermentation broth is accomplished by heat-treatment under acidic conditions, followed by the filtering of precipitated proteins and suspended particles of the culture medium. The antibiotic components are then separated and isolated by means of ion-exchange chromatography on a large column.

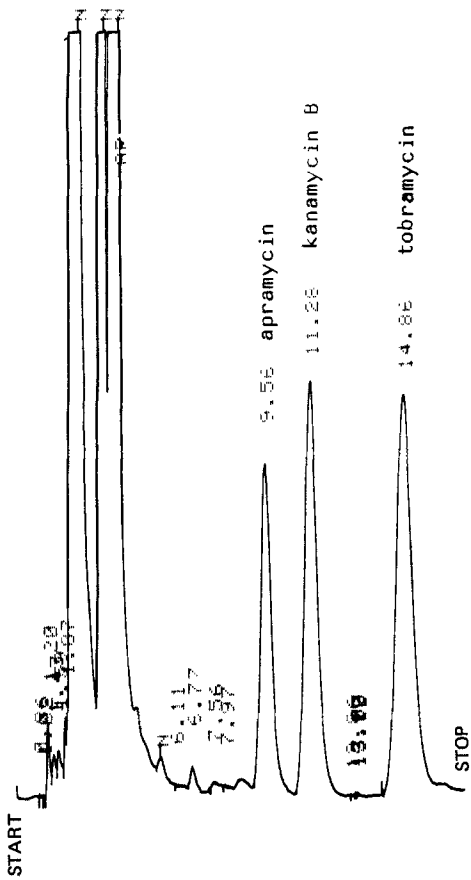


Fig. 3

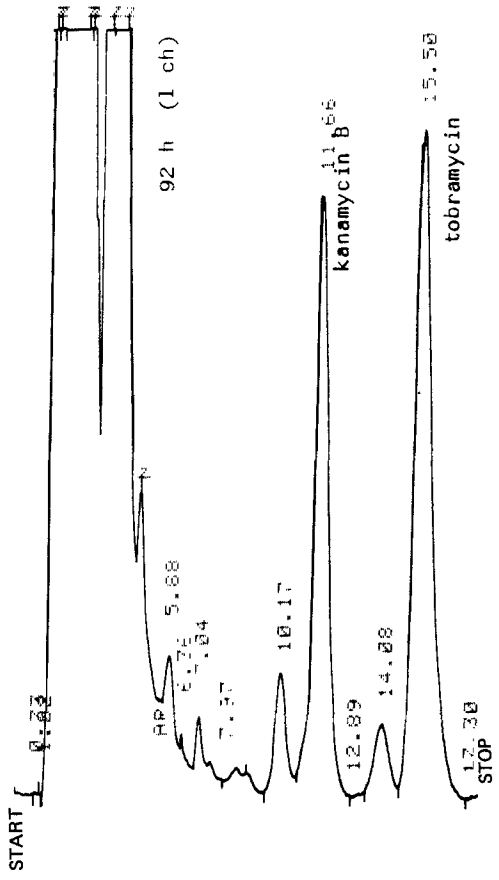


Fig. 4

Figures 3 & 4. Separation of apramycin, kanamycin B, and tobramycin on a reverse phase column from a mixture of the clear components (Fig. 3) and from a fermentation broth (Fig. 4).

For the liquid chromatographic analysis the removal of proteins could be achieved by treatment with tris-(hydroxymethyl)-aminomethane and subsequent centrifuging for a few minutes. Using this procedure several solid and colloid component of the system could be also removed.

The obtained clear fermentation broth was satisfactory for derivatization, i.e. for the determination of the antibiotic components (Fig. 4).

The analysis of these components in the fermentation broth allowed the monitoring of the antibiotic level upon fermentation. Taking samples in each 8th hour it was established that no detectable antibiotic substance is present after 24 hours of the inoculation of the producting strain (Streptomyces Tenebrarius). After that the antibiotic level quickly increases and reaches a maximum after 100-110 hours in the examined laboratory fermentations (Fig. 5). The fermentation has to be stopped and worked up at this point. Using this method the liquid chromatographic analysis, including the preparation of the samples, takes cca. 1.5 hours, so it is more quick and convenient than the microbiological evaluation.

EXPERIMENTAL

Preparation of the sample

To a 5 ml aliquot of the fermentation broth 5 ml aqueous solution of tris-(hydroxymethyl)-aminomethane (saturated) and 20 ml acetonitrile was added. The precipitated proteins were removed by centrifuging for 10 min. (at 3000 rpm) and the supernatant was decanted. For derivatization reaction 1 ml aliquot of this solution was used.

Preparation of the reference sample

To 100 ml of a "0 hr" fermentation broth the necessary amount of the antibiotics for the calibration was added

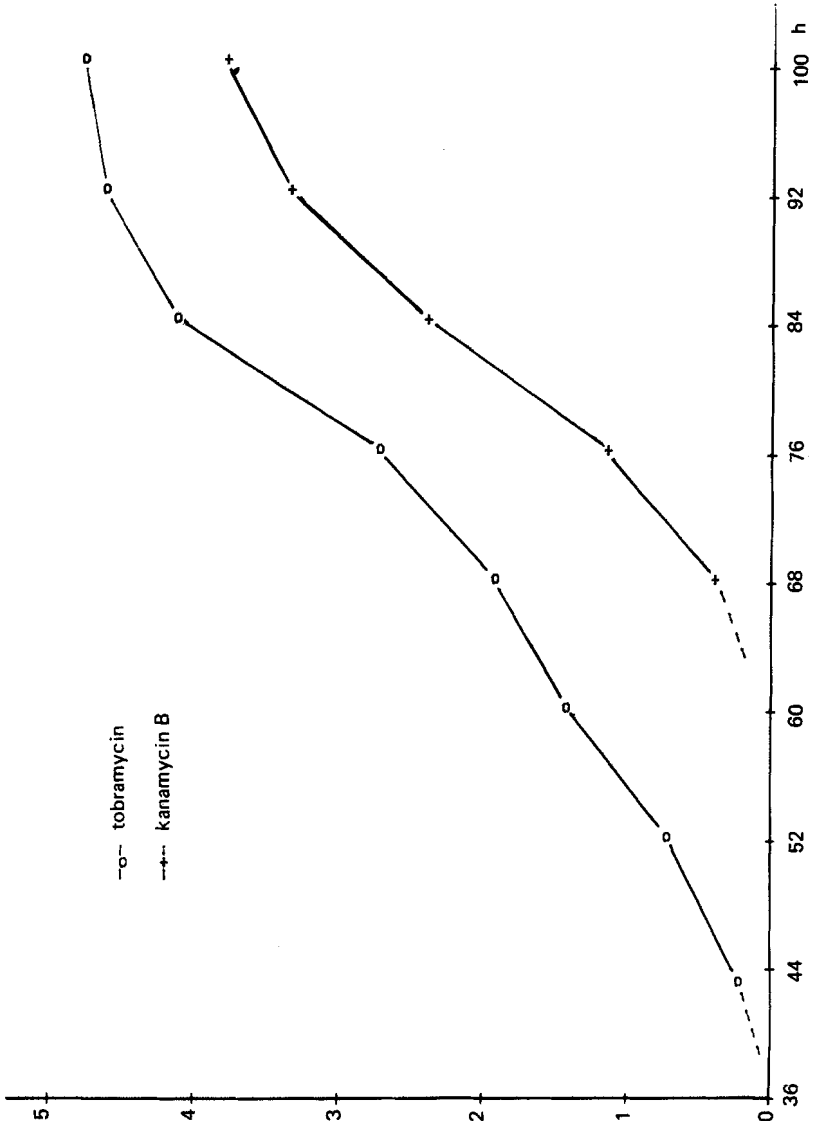


Figure 5. Concentration of the antibiotics during a fermentation. Apramycin was not detectable in this fermentation.

(apramycin: 0.2-1.0 mg, kanamycin B: 0.5-5.0 mg, tobramycin: 0.5-5.0 mg) and this solution was pre-treated similarly to that of the fermentation broth.

Derivatization

To 1 ml of a pre-threated fermentation broth 3 ml of 0.15 M methanolic DNFB solution was added. The mixture was heated at 100°C for 45 min. under reflux condenser, cooled, and the final volume of the solution was adjusted to 4 ml with eluent. This solution was applied for the determinations.

Liquid chromatographic determination

The analyses werw performed with a Hewlett-Packard 1081A isocratic instrument using a 20 ul loop Reodyne injector. The composition of the eluent was 55:45:0.15 acetonitrile-water-acetic acid, flow rate 1.2 ml/min. The separation was accomplished on a LiChrosorb RP-8 column (lenght: 20 cm, ID: 4.6 mm, particle size: 10 um, HP 79918B type). The detection was carried out at 350 nm, using an OE 308 type variable wave-lenght UV detector (Labor Műszeriapi Művek, Hungary). For the recording and integration a Hewlett-Packard 3385A type integrator was used. The quantitative determination was achieved according a calibration curve by measuring of the peak area.

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