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QUANTITATION OF NIKKOMYCINS IN BIOLOGICAL FLUIDS BY ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

In contrast to biological evaluation methods the use of high-performance liquid chromatography has allowed the quantitation of five nikkomycin components from the filtrate of the fermentation broth in which they are cultured.

INTRODUCTION

Nikkomycins (Fig. 1) are nucleoside–peptide antibiotics, similar in structure to polyoxins, and have been isolated from the culture broth of *Streptomyces tendae*[1–4]. These antibiotics are interesting from an agricultural point of view because they possess a high and selective antifungal and insecticidal activity. The site of action is associated with the biosynthesis of chitin in the cell wall[1,5]. An analytical method was then sought for the quantitation of the whole spectrum of these substances during the fermentation process. Biological testing using agar plate diffusion had disadvantages in that no quantitation of single components was possible, for although the antibiotically active substances could be determined the inactive substances could not and 2 days were necessary to evaluate the test plates using *Mucor hiemalis* as the test organism.

High-performance liquid chromatography (HPLC) is a powerful technique in quantitation of antibiotics in pharmaceutical analysis, but there have been few investigations of the use of this method for the control of antibiotic production. The quantitation of antibiotics from the fermentation broth by HPLC was reported in the cases of cephalosporin C[6,7], erythromycin and tetracyclin[8] and penicillins[9]. This paper demonstrates the use of HPLC for quantitation of five nikkomycin compounds from the culture filtrate of the fermentation broth.

EXPERIMENTAL

Chromatographic apparatus

The system consisted of a Spectra-Physics Model SP 8000-10 liquid chromatograph, a Spectra-Physics Model 8010 autosampler and a Schoeffel Model SF 770 variable-wavelength detector. A reversed-phase column packed with LiChrosorb

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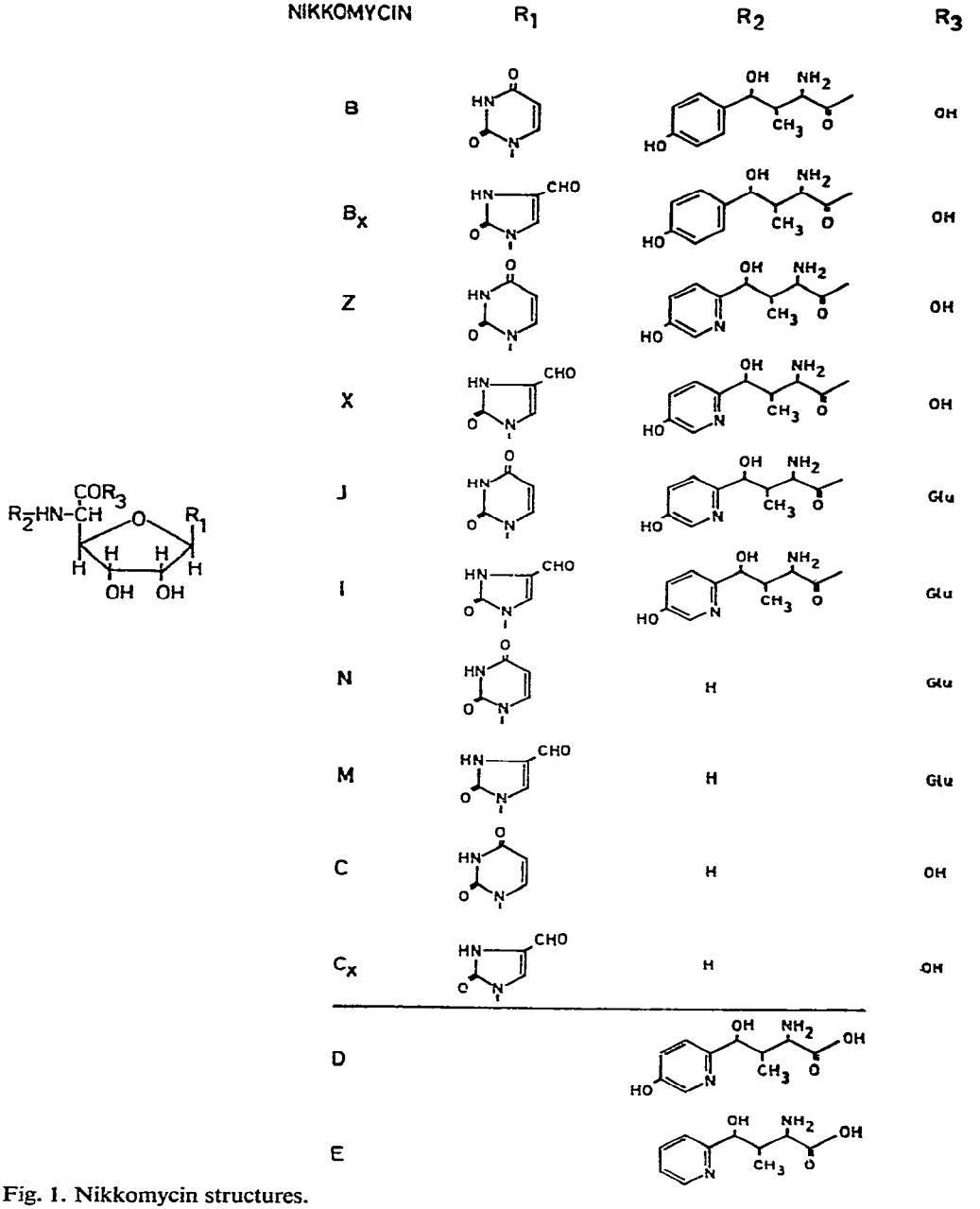


Fig. 1. Nikkomycin structures.

RP-8 (5 μm) of dimensions 120 × 4.6 mm I.D., supplied with a pre-column of dimensions 40 × 4.6 mm I.D., was obtained from Knauer (Berlin, G.F.R.).

For isocratic elution the mobile phase was 30 m*M* ammonium formate buffer, pH 4.7, containing 1 m*M* heptanesulphonic acid. The flow-rate was set to 1 ml/min and the pressure was approximately 100 bar. For gradient elution, solvent A was

30 m*M* ammonium formate buffer, pH 3.75, containing 1 m*M* heptanesulphonic acid, and solvent B was methanol containing 30 m*M* ammonium formate (corresponding to pH 3.75) and 1 m*M* heptanesulphonic acid. The flow-rate was set to 1 ml/min.

The detector wavelength was set at 290 nm and the sensitivity kept at 0.1 absorbance units full scale (a.u.f.s.).

Sample preparation

The fermentation broth was centrifuged and the supernatant containing the nikkomycins was filtered with a membrane filter (pore diameter 0.6 μm). A 10-μl sample was injected onto the column.

RESULTS AND DISCUSSION

Ion-pair reversed-phase chromatography seemed to be a promising method for separation of the nikkomycins because of the polar character of these substances and because quantitation has to be performed in an aqueous medium. Use of a mobile phase without a lipophilic counter-ion did not result in the separation of all nikkomycins. Ammonium formate buffer or ammonium acetate buffer, pH 4–6, separated nikkomycins C, D and I, but not Z and X, the last two components being eluted as one peak. The addition of a lipophilic basic counter-ion, such as tetramethyl-, tetraethyl- or tetrabutylammonium chloride, to the mobile phase did not result in the separation of all nikkomycins. When a lipophilic acidic counter-ion, such as dodecyl sulphate or heptanesulphonic acid, is included in the ammonium buffer, only with heptanesulphonic acid was the separation achieved of nikkomycins C, D, B, Z, X and I. A strong dependence was found between the pH of the mobile phase and the k' values, as shown in Table I.

Fig. 2 shows the separation of five nikkomycin standards and the HPLC analysis of a fermentation sample after a fermentation period of 145 h, under isocratic elution conditions. Fig. 3 demonstrates the separation of the standards using gradient elution.

The retention times and amounts recovered were reproducible whether isocratic or gradient elution was employed, as shown in Table II. Only the recovery of nikkomycin Z is unsatisfactory, because there is no baseline separation between Z and X, and therefore, Z was integrated as a rider peak.

When using gradient elution, only a short equilibration time under the initial conditions is necessary to achieve reproducible results. In isocratic elution the column must be equilibrated for 3 days after the regeneration with methanol in order to obtain reproducible results. On the other hand, when using isocratic elution conditions, the analysis time of approximately 18 min is shorter because of a 10 min initial delay in gradient elution, giving a total analysis time of 25 min.

Both methods are designed for the analysis of a great number of samples. Using isocratic elution, about 300 injections could be performed before regeneration of the column. When using a pre-column, about 3000 injections could be effected. However, it was necessary to change the pre-column after approximately 500 injections because of irreversible adsorption of substances from the culture filtrate on the stationary phase which resulted in poor separations.

TABLE I

CORRELATION BETWEEN pH OF THE AMMONIUM FORMATE BUFFER AND k' VALUES OF THE NIKKOMYCINS

Nikkomycin	*k' value*					
	pH 5.0	*4.9*	*4.8*	*4.7*	*4.6*	*4.5*
C	0.35	0.35	0.35	0.35	0.35	0.40
D	1.9	1.9	1.95	2.0	2.2	3.1
Z	3.2	3.35	3.5	3.65	4.8	6.5
X	3.65	3.8	4.0	4.2	5.6	7.5
I	5.8	6.8	9.3	10.7	19.7	30

Fig. 4 shows the quantitation of nikkomycins in the course of a fermentation period of 280 h, as well as biological testing, by HPLC analysis using isocratic elution conditions.

The advantages of HPLC are obvious. Several components could be detected in one run with a short analysis time. Not only the antibiotically active nikkomycins

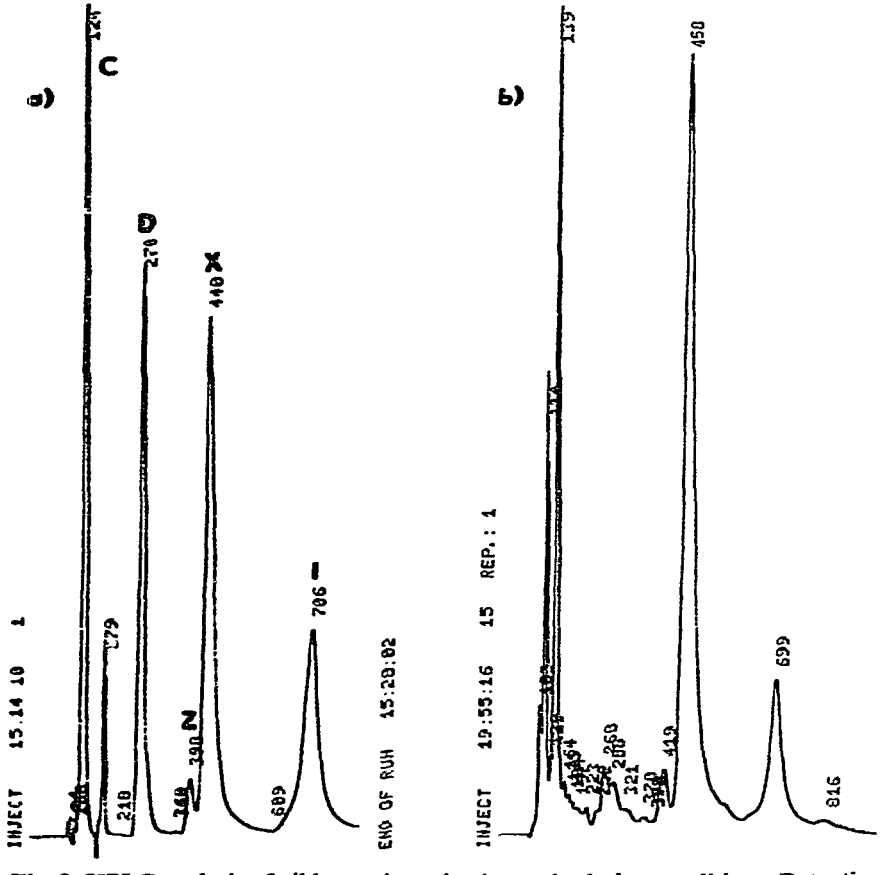
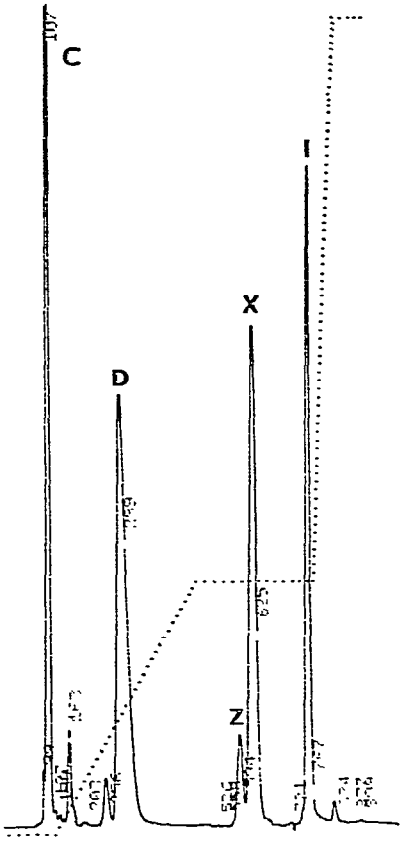


Fig. 2. HPLC analysis of nikkomycins using isocratic elution conditions. Retention times in seconds. a, Nikkomycin standard (1 mg/ml of each component); b, culture filtrate (fermentation period 145 h).



Gradientprofile

time (min)	% A	% B
0	100	0
2	100	0
8	70	30
13	70	30
14	0	100
15	0	100

Fig. 3. HPLC analysis of nikkomycin standards using gradient elution conditions. Retention times in seconds.

Z, X and I, but also the inactive components C and D could be quantitated. The amount of nikkomycin as determined by agar plate diffusion using *Mucor hiemalis* as the test organism corresponded to the sum of nikkomycins Z, X and I as determined by HPLC. Nikkomycins B/B_x were not produced under these fermentation conditions. Nikkomycins M and N are not biological products and therefore could not be

TABLE II

RELATIVE STANDARD DEVIATIONS (%)

Nikkomycin	*Isocratic elution*		*Gradient elution*	
	Retention time	*Concentration*	*Retention time*	*Concentration*
C	0.45	1.25	1.5	1.25
D	0.55	1.39	1.0	1.24
Z	0.85	8.52	0.66	6.3
X	0.88	1.50	0.3	2.36
I	1.14	2.52	1.0	2.51

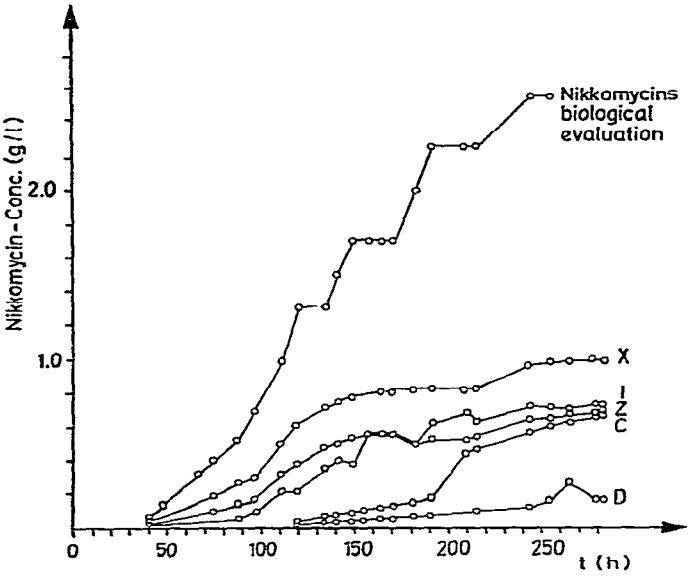


Fig. 4. Production control of nikkomycins in the course of a fermentation cycle.

detected in the fermentation broth. Both substances result from chemical hydrolysis of nikkomycins I and J.

The limit of detection using absorption spectrophotometry at 290 nm was approximately 0.05 μg of each component.

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REFERENCES

1 U. Dähn, H. Hagenmaier, H. Höhne, W. A. König, G. Wolf and H. Zähner, *Arch. Microbiol.*, 107 (1976) 143.
2 H. Hagenmaier, A. Keckeisen, H. Zähner and W. A. König, *Justus Liebigs Ann. Chem.*, (1979) 1494.
3 H. Hagenmaier, A. Keckeisen, W. Dehler, H.-P. Fiedler, H. Zähner and W. A. König, *Justus Liebigs Ann. Chem.*, in press.
4 W. A. König, W. Hass, W. Dehler, H.-P. Fiedler and H. Zähner, *Justus Liebigs Ann. Chem.*, (1980) 622.
5 G. U. Brillinger, *Arch. Microbiol.*, 121 (1979) 71.
6 R. D. Miller and N. Neuss, *J. Antibiot.*, 29 (1976) 902.
7 R. D. Miller and N. Neuss, *J. Antibiot.*, 31 (1978) 1132.
8 K. Tsuji and J. F. Goetz, *J. Antibiot.*, 31 (1978) 302.
9 F. Nachtmann, *Chromatographia*, 12 (1979) 380.