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Note

Niphimycin

III. Chromatographic separation of the components of the niphimycin complex

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It has been proven by Blinov *et al.*[1] that niphimycin contains two biologically active components that can be separated by paper chromatography with butanol saturated with water. It was subsequently shown[2,3] that each of these two components (designated by Againa and Ognianov[3] as niphimycin A and B) can be separated into two further substances by thin-layer chromatography (TLC) in the system chloroform–methanol–water (2:2:1). Therefore, niphimycin contains four components, designated as niphimycin A_1, A_2, B_1 and B_2, as well as certain small amounts of impurities. The components A_1 and A_2 represent the main part of the niphimycin complex. The four components are very similar in their chromatographic behaviour, which creates difficulties in achieving good results in their chromatographic separation.

This paper describes the separation of the niphimycin components and their antimicrobial properties.

SEPARATION OF NIPHIMYCIN A AND B BY PREPARATIVE THIN-LAYER CHROMATOGRAPHY

Pre-coated plates of Kieselgel GF_{254} (Merck, Darmstadt, G.F.R.) were used. Amounts of 8–10 mg of the antibiotic dissolved in methanol were applied on a 20 × 20 cm plate and the chromatogram was developed twice in the system ethyl acetate–ethanol–water (150:45:28) or *n*-butanol–ethanol–water (4:1:5) (upper phase). The spots, detected with a UV lamp, were eluted with methanol and dried under vacuum, and the dried material was dissolved in methanol, filtered and precipitated with acetone or diethyl ether.

SEPARATION OF NIPHIMYCIN A AND B ON A CELITE COLUMN

Celite 535 (100 g) was suspended in ethyl acetate and poured into a glass column 3.5 cm in diameter, then 1 g of niphimycin complex dissolved in 10 ml of methanol was added to 10 g of Celite and dried under vacuum. After being suspended in ethyl acetate, the material was applied on to the column. Elution was carried out with the system ethyl acetate–90% ethanol (30:4). Fractions of 50 ml were collected,

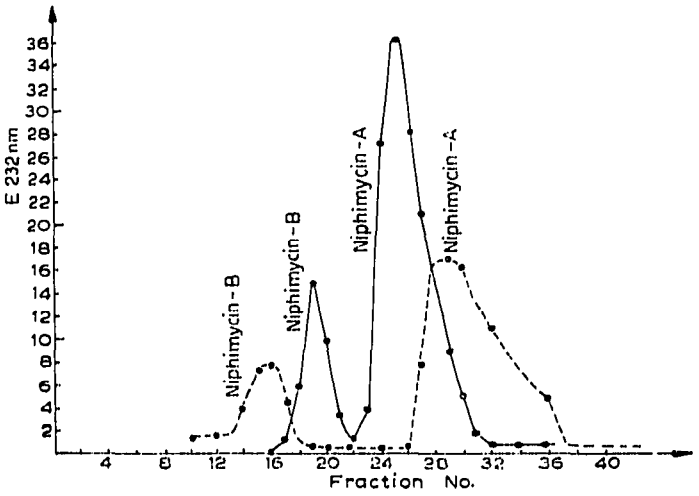

Fig. 1. Elution of niphimycin complex on Sephadex LH-20. Bed dimensions: 1.7 × 50 cm. Flow-rate: 3.5 ml/h. Broken line: sample, 1.5 ml containing 30 mg of niphimycin complex; solvent, 30% ethanol. Solid line: sample, 1.5 ml containing 40 mg of niphimycin complex; solvent, 35% ethanol.

each being analyzed by TLC in the system *n*-butanol–ethanol–water (4:1:5) (the upper phase being used). Fractions 5–11 contained niphimycin A, while fractions 36–43 contained niphimycin B. The pooled eluates containing only component A or B were evaporated to dryness under vacuum and the material obtained was extracted with methanol, filtered and precipitated with acetone. The results given in Table I demonstrate that niphimycin A is eluted within a considerably narrow fraction interval in a satisfactory high yield, while niphimycin B is eluted predominantly as a mixed fraction in low yield.

SEPARATION OF NIPHIMYCIN A AND B BY COLUMN CHROMATOGRAPHY

A glass column (5 × 100 cm) packed with Sephadex LH-20 suspended in 30% aqueous acetone was used. A 700-mg amount of niphimycin complex was dissolved in 7 ml of 30% acetone and applied on to the column. Elution was carried out with the above solvent at a flow-rate of 36 ml/h; 140 fractions of 18 ml were collected. All of the fractions were analyzed by TLC as described above. Fractions 60–71 contained niphimycin B, while niphimycin A was present in fractions 84–138. The eluates containing niphimycin B were concentrated to one eighth of the initial volume at 35° under vacuum and then freeze-dried. Part of the fractions containing niphimycin A (90–119) precipitated after being kept in a refrigerator overnight. The white precipitate was filtered, washed with water and dried. On the other hand, the filtrate was pooled with the remaining fractions (84–138), concentrated under vacuum and freeze-dried.

The results in Table I show that a good separation of the two components (A and B) can be achieved by chromatography on Sephadex LH-20. Also, eluates of high purity that in the cold produce a precipitate containing mainly niphimycin A_1

and a small proportion of niphimycin A_2 can be obtained. In the native mixture, these products are present in approximately equal amounts. The TLC analysis of the freeze-dried product A shows that it is rich in niphimycin A_2.

The separation of niphimycin A and B on Sephadex LH-20 was also achieved by elution with aqueous alcohols. Fig. 1 shows the elution diagrams of niphimycin complex after filtration on Sephadex LH-20. Elution was carried out with aqueous alcohol and all of the fractions were analyzed by TLC as described above, the extinction being measured at 232 nm. It can be seen that the elution peaks correspond to niphimycin B and A. The appropriate concentration of the eluent and the amount of the sample applied are essential for the effectiveness of the separation. The peaks of niphimycin A and B obtained from 30 mg of starting material and eluted with 30% ethanol were separated more effectively than when 40 mg of the sample were applied and 35% ethanol was used as eluent. Separation was not achieved when aqueous acetone or ethanol at concentrations higher than 35% were used. Niphimycin A and B obtained by the methods described were subjected to chromatography on Kieselgel in order to fractionate components A_1 and A_2 or B_1 and B_2.

CHROMATOGRAPHIC FRACTIONATION OF NIPHIMYCIN A ON KIESELGEL

A 200-g amount of Kieselgel (Merck) was suspended in the lower phase of the system chloroform–methanol–water (35:20:10) and poured into a column 2.4 cm in diameter. Niphimycin A (400 mg) obtained by one of the methods described above was dissolved in 5 ml of methanol and mixed with 8–10 g of Kieselgel. After being dried, the mixture was suspended in the above system and applied on to the column. Elution was carried out at a flow-rate of 5 ml/min and fractions of 100 ml were collected. All of the fractions were analyzed by TLC using the lower phase of the mixture chloroform–methanol–water (2:1:1)[3]. The fractions of the corresponding peaks were pooled and treated as described under Separation of niphimycin A and B on a Celite column. The results are given in Table I.

TABLE I

CHROMATOGRAPHIC SEPARATION OF THE NIPHIMYCIN COMPLEX

Method	*Starting material*	*Component*	*Fraction Nos.*	*Weight (mg)*
Column chromatography on Celite	Niphimycin complex (1 g)	A	5– 11	562
		A + B	12– 36	124
		B	37– 43	43
Column chromatography on Sephadex LH-20	Niphimycin complex (700 mg)	A	60– 71	86
		A + B	—	—
		B	84–138	259 precipitate + 293 freeze-dried material
Kieselgel column chromatography	Niphimycin A (400 mg)	A_1	25– 29	115
		A_1 + A_2	30– 35	108
		A_2	35– 47	92
Kieselgel column chromatography	Niphimycin B (150 mg)	B_1	102–115	54
		B_1 + B_2	116–129	32
		B_2	130–164	18

TABLE II

MINIMAL INHIBITORY CONCENTRATIONS ESTABLISHED FOR THE DIFFERENT COMPONENTS OF NIPHIMYCIN (μg/ml)

Sample	*Microorganism*			
	Candida albicans	*Trichophyton gypseum*	*Fusarium graminarum*	*Bacillus subtilis*
Niphimycin complex	1.56–3.12	2.34–3.12	2.34–3.12	6.25
Niphimycin A	1.56	1.56–3.12	3.12	6.25
Niphimycin A_1	1.56	3.12	3.12	6.25
Niphimycin A_2	7.80	15.6	7.8	31.25
Niphimycin B	3.12	6.25	3.12	12.5
Niphimycin B_1	1.56	1.56	3.12	—
Niphimycin B_2	1.56	3.12	3.12	—

CHROMATOGRAPHIC FRACTIONATION OF NIPHIMYCIN B ON KIESELGEL

A column packed with 150 g of Kieselgel (Merck) was prepared as described above. Niphimycin B (150 mg) was dissolved in methanol and mixed with 3 g of Kieselgel and the suspension was dried under vacuum and applied on to the column. Elution was carried out with chloroform–methanol–water (35:20:10). Fractions of 20 ml were collected and analyzed in the system chloroform–methanol–water (2:1:1) as described in the previous section. The pooled, evaporated and dried fractions were extracted with methanol and precipitated with diethyl ether. The results are given in Table I.

ANTIMICROBIAL PROPERTIES

The antimicrobial action of the four niphimycins was studied by the method of serial dilution using *Candida albicans*, *Trichophyton gypseum*, *Fusarium graminarum* and *Bacillus subtilis*. The results obtained (Table II) demonstrate that there is no substantial difference in the spectrum of action of the separate niphimycins and the niphimycin complex. *In vitro* they have a high fungicidal effect and a smaller antibacterial activity.

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