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Comparative Study of Responses to Neomycins B and C by Microbiological and Gas-Liquid Chromatographic Assay Methods

KIYOSHI TSUJI, JOHN H. ROBERTSON, RUTH BAAS, AND D. J. McINNIS

Biological Research and Development, Microbiological Product Control, The Upjohn Company, Kalamazoo, Michigan 49001

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The relative responses of neomycins B and C have been determined by a microbiological agar-diffusion method, a turbidimetric method, and by a recently developed gas-liquid-chromatographic (GLC) method capable of separating the neomycin isomers. The ratios of response of neomycin C to neomycin B by the individual methods were as follows: agar-diffusion method, 1:3; turbidimetric method, 1:2.5; and GLC method, 1:1. When neomycin C is assumed to have 35% biological activity of neomycin B, the calculated drug contents of neomycin sulfate powders obtained by the GLC method correlated well with values obtained by the microbiological agar-diffusion assay method.

Neomycin, as defined in the Code of Federal Regulations (2), is "each of the antibiotic substances produced by *Streptomyces fradiae*, and each of the same substances produced by any other means." The antimicrobial components of neomycin (Fig. 1) include neamine (neomycin A) and neomycins B, C, LP_B, and LP_C (10; W. S. Chilton, Ph.D. Thesis, Univ. of Illinois, Urbana, 1963). The antimicrobial activity of these components drops in the order of neomycin B to C to neamine. Neomycins LP_B and LP_C possess low antimicrobial activity (W. S. Chilton, 1963), but data are not available as to their activities in relation to neomycin B, neomycin C, or neamine. Since the ratio of these components varies from lot to lot, the drug content of one particular lot, as determined by the microbiological assay method, depends on the ratio of the components in the sample and the reference standard. Also, the response by a microbiological method to each of these components is quite often variable (10).

There are several chemical methods which are capable of quantitating neomycins B and C (1, 4-6, 9). However, these methods are time-consuming and are not suitable for a laboratory in which a large number of neomycin products are quantitated routinely. The gas-liquid-chromatographic (GLC) method described by Tsuji and Robertson (12), on the other hand, enables quantitation of neomycins B and C with greater facility than any other method.

The purpose of this paper is to compare the responses of neomycins B and C by the GLC and microbiological assay methods, thereby correlating drug content.

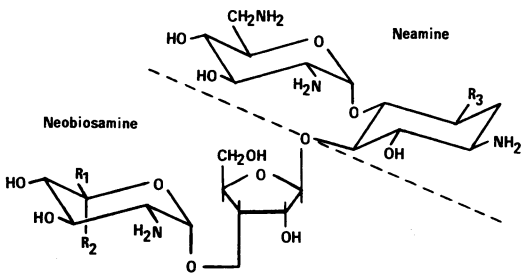
MATERIALS AND METHODS

Agar-diffusion method. The test microorganism was *Staphylococcus aureus* ATCC 6538P. The assay medium was Trypticase Soy Seed Agar (BBL). The method described in the Code of Federal Regulations (2) was used.

Turbidimetric method. The test microorganism was *Klebsiella pneumoniae* ATCC 10031. The assay medium was Antibiotic Assay Broth (BBL). The general turbidimetric assay procedure was used as described by Kirk (7).

GLC method. The method used was developed by Tsuji and Robertson (12). It is based on the silylation of neomycin with *N*-trimethylsilyldiethylamine (Pierce Chemical Co,. Rockford, Ill.) in Tri-Sil "Z" (Pierce Chemical Co.). Trimyristin or trilaurin (Supelco, Inc., St. Bellefonte, Pa.) may be used as an internal standard. However, trilaurin is the internal standard of choice, since the chromatographic retention time of neomycin LP_B is similar to that of trimyristin. Silylated neomycin was chromatographed on an 0.75% OV-1 (Applied Science Laboratory, State College, Pa.) column (3 by 1,830 mm, glass) at 290 C, taking approximately 30 min per sample.

Sample preparation. Aqueous solutions of neomycin B (USP lot I reference standard) and neomycin C were prepared to contain 10 mg/ml. The two neomycin solutions were then mixed in proportions of 20, 50, and 80%. Solutions thus prepared were then diluted to 10 μg/ml and stored frozen in liquid nitro-



Neomycin B	R_1=H	R_2=CH_2NH_2	R_3=NH_2
Neomycin C	R_1=CH_2NH_2	R_2=H	R_3=NH_2
Neomycin LP_B	R_1=H	R_2=CH_2NH_2	R_3=$NHCOCH_3$
Neomycin LP_C	R_1=CH_2NH_2	R_2=H	R_3=$NHCOCH_3$

FIG. 1. *Structure of neomycin.*

TABLE 1. *Relative responses of neomycins B and C by GLC, agar-diffusion, and turbidimetric methods*

Method[a]	Neomycin B (%) 100	80	50	20	0
	Neomycin C (%) 0	20	50	80	100
GLC					
Response.....	100	101.4	99.3	101.6	101.2
Expected response[b].....		100.2	100.6	100.9	
Neomycin C found (%)...	0	23.4	54.3	83.9	100
Turbidimetric					
Response.....	100	87.8	68.9	50.9	38.9
Expected response[b].....		87.8	69.5	51.1	
Agar-diffusion					
Response.....	100	92.2	66.2	47.0	33.6
Expected response[b].....		86.7	66.8	46.9	

[a] The coefficient of variation was 1.3 for the GLC method, 1.2 for the turbidimetric method, and 3.9 for the agar-diffusion method.

[b] Expected response = (neomycin B fraction) × (neomycin B response) + (neomycin C fraction) × (neomycin C response).

gen until they were assayed microbiologically. For the gas chromatographic determination, the neomycin solutions were diluted to 6 mg/ml. One-milliliter amounts of the solutions were pipetted into 1.5-ml serum vials and freeze-dried. The vials were then capped with 13-mm, natural red rubber closures and stored in a desiccator over silica gel until assayed. Samples were submitted in double blind fashion to each of three analysts and were assayed on 3 consecutive days.

The USP lot I reference standard (767 μg of neomycin base per mg of anhydrous neomycin sulfate) was used to calculate the neomycin content of samples.

RESULTS AND DISCUSSION

Responses of neomycins B and C. The relative response of neomycin C to neomycin B (Table 1) was lowest by the agar-diffusion method (34%), followed closely by the turbidimetric method (39%). This ratio of biological response, however, is not always constant. The variability experienced over the years is as follows: agar diffusion method, 30 to 36%; turbidimetric method, 33 to 40%. Factors which contribute to the variability in microbiological response were discussed by Sokolski et al. (11). Freyburger and Johnson (3) also reported that different microorganisms respond differently to neomycins B and C. Since the definition of neomycin includes all of the neomycin entities (2), the ideal assay method should have equal response to neomycins B and C, as does the GLC method.

TABLE 2. *Drug content of neomycin powder*

Sample	GLC assay				Agar-diffusion assay
	Neamine	Neomycin C	Total neomycin[a]	Calculated microbial response[b]	
	%	%	μg/mg	μg/mg	μg/mg
1	0	9.8	707	662	659
2	0	11.6	717	663	660
3	0	33.6	760	594	568

[a] Neomycins B and C.

[b] Antimicrobial activity of neomycin C is assumed to be 35% of neomycin B.

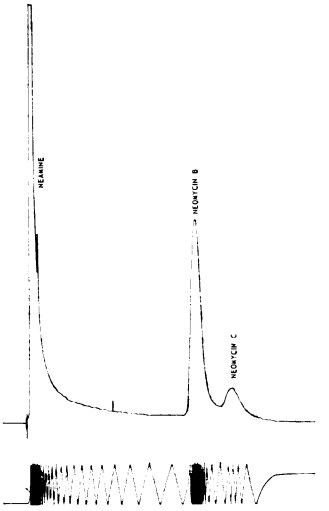


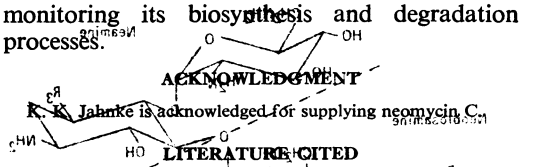
FIG. 2. *Chromatogram of neomycin international reference standard indicating separation of neamine, neomycin B, and neomycin C.*

The method is capable of separation and quantitation of neomycins B and C in approximately 30 min (Fig. 2). The neomycin C content of samples detected by the GLC method is listed in Table 1. The slight positive bias in the neomycin C content may be due to an incomplete separation of neomycin B and C peaks. The expected responses, as calculated by the following formula, agree well with the responses obtained by the three methods, with the exception of the agar-diffusion method at the 20% neomycin C level.

The expected response equals $B_f \times B_r + C_f \times C_r$, where B_f is the neomycin B fraction of a given sample, B_r is the neomycin B response at its 100% level, C_f is the neomycin C fraction of a given sample, and C_r is the neomycin C response at its 100% level. The coefficient of variation was 1.3% for the GLC method, 1.2% for the turbidimetric method, and 3.9% for the agar-diffusion method.

Quantitative data. The neomycin content of three samples, quantitated by both the GLC and the agar-diffusion methods, is listed in Table 2. The data by the GLC method indicate that these powders contain 9.8, 11.6, and 33.6% of neomycin C; however, neamine, neomycin LP_B and neomycin LP_C were not detected. From these data, probable microbiological responses were calculated by assuming the antimicrobial activity of neomycin C to be 35% of neomycin B. The calculated microbiological responses, thus obtained agree well with values obtained by the agar-diffusion method.

Thus, the GLC method should be a valuable tool for the quantitation of neomycin and for monitoring its biosynthesis and degradation processes.



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