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

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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, LPB, and LPC (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 LPB and LPC 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 Broth (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.). Trimyrystin 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 LPB is similar to that of trimyrystin. 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-


FIG. 1. Structure of neomycin.

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

<table>
<thead>
<tr>
<th>Method*</th>
<th>Neomycin B (%) 100</th>
<th>80</th>
<th>50</th>
<th>20</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neomycin C (%) 0</td>
<td>20</td>
<td>50</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>GLC</td>
<td>Response . . . . .</td>
<td>100</td>
<td>101.4</td>
<td>99.3</td>
<td>101.6</td>
</tr>
<tr>
<td></td>
<td>Expected response b</td>
<td>100.2</td>
<td>100.6</td>
<td>100.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neomycin C found (%)</td>
<td>0</td>
<td>23.4</td>
<td>54.3</td>
<td>83.9</td>
</tr>
<tr>
<td></td>
<td>Turbidimetric . . .</td>
<td>100</td>
<td>87.8</td>
<td>68.9</td>
<td>50.9</td>
</tr>
<tr>
<td></td>
<td>Expected response .</td>
<td>87.8</td>
<td>69.5</td>
<td>51.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agar-diffusion . . .</td>
<td>100</td>
<td>92.2</td>
<td>66.2</td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td>Expected response .</td>
<td>86.7</td>
<td>66.8</td>
<td>46.9</td>
<td></td>
</tr>
</tbody>
</table>

* 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) x (neomycin B response) + (neomycin C fraction) x (neomycin C response).

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Neamine</th>
<th>Neomycin C</th>
<th>Total neomycin</th>
<th>Calculated microbial response</th>
<th>Agar-diffusion assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>%</td>
<td>%</td>
<td>µg/mg</td>
<td>µg/mg</td>
<td>µg/mg</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>9.8</td>
<td>707</td>
<td>662</td>
<td>659</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>11.6</td>
<td>717</td>
<td>663</td>
<td>660</td>
</tr>
</tbody>
</table>

* 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 merit of the GLC method is its capability 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 LPB and neomycin LP C were not detected. From these data, probable microbiological responses were calculated by assuming the antibacterial 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.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**