Comparison of Enzymatic and Microbiological Gentamicin Assays

ARNO LD. SMITH, J. ALLAN WAITZ, DAVID H. SMITH, EDWIN M. ODEN, AND BARBARA B. EMERSON

Division of Infectious Diseases, Children's Hospital Medical Center, Boston, Massachusetts 02115, and the Chemotherapy Department, Schering Corporation, Bloomfield, New Jersey 07003

Received for publication 28 March 1974

The correlation coefficient between the rapid enzymatic and the overnight microbiological assays for 211 urine and serum specimens was 0.96. The 95% confidence limits yielded a correlation coefficient between 0.92 and 0.98. Both methods tended to underestimate the amount of a gentamicin added to urine. When only serum samples were considered, the predicted value obtained from the linear regression analysis of either method was within 0.57 µg/ml 99% of the time. This high degree of positive correlation will permit safe rapid adjustment of individualized patient gentamicin dosages.

Since gentamicin is excreted primarily through the kidneys, hazardous concentrations can accumulate in patients with renal failure (2). Therefore, there are many indications for the determination of blood gentamicin concentration. Rapid assays have been developed to allow close monitoring of individual patients (1, 3, 4, 10). These include spore-inoculated agar plates (7), radioimmunoassay (1, 3), enzymatic (8), and aminoglycoside inhibition of the metabolic activity of microorganisms (4). The reference assay systems have been the overnight Staphylococcus aureus and the Bacillus subtilis spore methods. Before one of the rapid assays can be routinely used for patient management, its accuracy and precision relative to the reference methods must be known. This report details the comparison of the overnight assay technique (5) (S. aureus) with the enzymatic method (8).

MATERIALS AND METHODS

Specimen. Sixty-nine samples were mock unknowns constructed by the addition of a known quantity of gentamicin to serum from hospitalized patients not receiving gentamicin. Fifty-two of these samples were derived from patients not receiving any antimicrobial agents; of these, 23 were from patients with abnormal renal function (blood urea nitrogen greater than 50 mg%) or abnormal hepatic function (bilirubin greater than 3 mg% and serum glutamic oxalacetic transaminase greater than 100 IU), or both. The remaining 17 samples contained chloramphenicol, ampicillin, nafcillin, oxacillin, or neomycin alone or in combination. One hundred and twenty-three serum specimens were obtained from patients receiving parenteral gentamicin and usually a semisynthetic (type I penicillinase-resistant) penicillin. Nineteen urine samples were constructed by the addition of known amounts of gentamicin to the urine of healthy adult male volunteers. All specimens were frozen at −20 C after collection until assay.

Microbiological assay. Gentamicin was assayed in triplicate by the method previously described (5). Sterile 6.35-mm paper disks were dipped in the specimen, shaken to remove the excess, and placed on agar plates previously seeded with S. aureus negative forms (ATCC 6538 P). After overnight incubation (usually 18 h) at 37 C, the zone diameter of the samples was compared with that of standards prepared in pooled normal human serum using gentamicin of known potency. All samples were preincubated with type II β-lactamase (Whatman Associates).

Enzymatic assay. The enzymatic assay was modified from the original description (9) as described (8). All samples were heated for 5 min at 60 C to inactivate contained enzyme activity capable of destroying adenine triphosphate (ATP). Ten microliters of sample or standard was then added to 25 µl of partially purified gentamicin adenyltransferase (GAT) and 75 µl of assay mix containing: 0.450 pmol of [14C]ATP (specific activity, 65,821 counts per min per nmol), 0.2 nmol of MgCl2, 0.9 nmol of tris(hydroxymethyl)aminomethane-HCl (pH 8.6), 1 nmol of dithiothreitol, and 4.5 pmol of ATP (cold); and incubated 1 h at 35 C. After incubation, 75 µl was removed, spotted on Whatman P-81 phosphocellulose squares (25 by 25 mm), washed for 20 min in 5 mM tris(hydroxymethyl)aminomethane-Cl (pH 7.4), and dried, and the bound radioactivity was determined by liquid scintillation spectrometry. Standards were prepared in the serum of one of us (A.L.S.) by using gentamicin powder of known potency. Gentamicin concentrations were calculated by comparison of the radioactivity obtained with the standards to that obtained with the samples. The average slope of 43 consecutive standard curves prepared over a 5-month period was 870 counts/min per 1 µg/ml.
RESULTS

Figure 1 depicts the gentamicin concentrations obtained on 211 samples. The arithmetic sample correlation coefficient between the two methods was \( r = 0.96 \). Within 95% confidence limits, the population correlation coefficient \( \rho \) was 0.92 to 0.98; i.e., 95% of the values had a correlation coefficient lying between 0.92 and 0.98 for the samples. (The equation for the regression line was \( y = a + bx \).) The slope, \( \beta \), was 1.40, and the intercept (the value of \( y \) when \( x = 0 \)), \( \alpha \), was -1.5 for these 211 samples. The negative value of \( \alpha \) was due to skewing of the regression line by the somewhat poorer correlation of the methods at high gentamicin concentrations. This effect can be demonstrated by the calculation of the logarithmic correlation coefficient. The value of \( r \) did not change appreciably (0.95), but the values of \( \beta (1.03) \) and \( \alpha (-0.002) \) were much nearer to perfect correlation.

The statistical data obtained when the samples were truncated at 20 \( \mu g/ml \) is shown in Table 1. This manipulation did not improve the arithmetic correlation coefficient. This coefficient was probably a reflection of the 100-fold range (0.2 to 20 \( \mu g/ml \)) of the samples even after exclusion of non-physiological samples. The logarithmic correlation coefficient, which unweights the extremely high and low concentrations, remained close to the ideal after truncation of the samples at 20 \( \mu g/ml \).

The important issue is the correlation between the two methods for serum specimens, particularly when other antimicrobials are present. There was good correlation \( (r = 0.96) \) between the two methods in the assay of 192 serum samples, two-thirds of which were derived from clinical studies (Table 1). Although there was a good correlation between the two methods, \( r \), the correlation coefficient, reflected only the linear trend. The standard error of the estimate, however, gave a description of a subpopulation of the values with one method at a given concentration when a value obtained with the other was used for predictive purposes. Thus, the standard error of the estimate \( (\sigma_y) \) of the microbiological method \( (y) \) at a given concentration in serum determined by the enzymatic technique \( (x) \) was 0.190 \( \mu g/ml \). This means that when the value obtained by the enzymatic method on a serum sample is used to predict that which will be obtained by the microbiological method on the basis of the linear regression, the value predicted will be ±0.38 \( \mu g/ml \) 95% of the time and ±0.57 \( \mu g/ml \) 99% of the time. Thus the information gained from knowing \( \sigma_y \) will allow the recognition of extraneous (non-methodological) influences in the assays. For example, a 10 \( \mu g/ml \) difference between the two methods with a single sample would suggest that there are compounds other

FIG. 1. Comparison of the gentamicin concentration obtained by duplicate enzymatic and quadruplicate microbiological assay of 211 sera and urine samples containing gentamicin.

### Table 1. Statistical data obtained when comparing the enzymatic and microbiological gentamicin assay methods

<table>
<thead>
<tr>
<th>Source of samples</th>
<th>Type of calculation</th>
<th>( N )</th>
<th>( a )</th>
<th>( \beta )</th>
<th>( r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum and urines truncated at 20 ( \mu g/ml )</td>
<td>Arithmetic</td>
<td>172</td>
<td>0.408</td>
<td>0.974</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Logarithmic</td>
<td>172</td>
<td>0.023</td>
<td>1.04</td>
<td>0.94</td>
</tr>
<tr>
<td>Mock unknowns in urine</td>
<td>Arithmetic</td>
<td>19</td>
<td>-1.93</td>
<td>1.24</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Logarithmic</td>
<td>19</td>
<td>0.39</td>
<td>0.79</td>
<td>0.93</td>
</tr>
<tr>
<td>Enzymatic ((x)) vs. theoretical ((y))</td>
<td>Arithmetic</td>
<td>19</td>
<td>3.30</td>
<td>1.00</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Logarithmic</td>
<td>19</td>
<td>0.39</td>
<td>0.75</td>
<td>0.93</td>
</tr>
<tr>
<td>Microbiological ((x)) vs. theoretical ((y))</td>
<td>Arithmetic</td>
<td>19</td>
<td>5.68</td>
<td>0.78</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>Logarithmic</td>
<td>19</td>
<td>0.13</td>
<td>0.91</td>
<td>0.98</td>
</tr>
<tr>
<td>Serum samples (enzymatic vs. microbiological)</td>
<td>Arithmetic</td>
<td>192</td>
<td>-1.73</td>
<td>1.48</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>Logarithmic</td>
<td>192</td>
<td>-0.027</td>
<td>1.07</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* Abbreviations: \( N \), number of samples; \( a \), \( y \) intercept on the \( x \) axis; \( \beta \), slope; \( r \), linear correlation coefficient.
than gentamicin that are being determined as gentamicin.

Mock unknowns prepared in urine ranged from 1.0 to 120.0 µg/ml, with 10 of the samples containing less than 20 µg/ml. The statistical data obtained from correlations between the methods with urine samples is depicted in Table 1. Although the value of $r$ was good with either method of correlation (0.93 arithmetic and 0.97 logarithmic), the slopes ($\beta$) and intercepts ($\alpha$) indicated a consistent trend to obtain higher concentrations with the microbiological method. This trend did not appear to be due to the microbiological method detecting non-antibiotic antimicrobial substances in urine, since both methods correlated well with the theoretical (calculated) amount of gentamicin present. Both methods, in general, tended to underestimate the amount of gentamicin present at high concentrations.

**DISCUSSION**

Gentamicin is inactivated as an antibiotic by adenylate, catalyzed by GAT. Although there are no published studies with gentamicin adenylate, one might infer that the moiety of the antibiotic recognized by GAT is the same as that recognized by the ribosome of bacteria. Thus, in a microbiological assay in which the end point is the presence or absence of bacterial growth, the basic action of gentamicin is the inhibition of ribosomal protein synthesis. It is not surprising, then, that there is a good correlation between the microbiological (ribosomal recognition) and enzymatic (GAT recognition) assay for gentamicin. The other alternative is that gentamicin adenylate has the ribosomal site open but unavailable for binding because of steric interference. This does not negate the correlation, because growth of bacteria still depends on protein synthesis. If, however, there are other agents (antibiotics) present that will inhibit bacterial growth, then the microbiological assay will produce falsely high gentamicin values. In practice, this obstacle is overcome by inactivating the antibiotics or by using an indicator organism resistant to the antibiotics simultaneously present. Non-antibiotic antimicrobial substances are difficult to anticipate and can be a continuing source of error with a biological assay.

Likewise the presence of cationic substance that can be adenylated by GAT will give falsely high gentamicin values. At this time, the only substances that can serve in this capacity are the related antibiotics tobramycin and kanamycin (8). Because of the similarities in antimicrobial spectrum, pharmacokinetics, and toxicity, one would not anticipate their simultaneous use with gentamicin. However, if tobramycin and kanamycin are present, the observed gentamicin value will be in error.

In this institution, if we consider the cost of the reagents, technician wages, and overhead, and prorate the equipment over a 12-year period, the cost of each enzymatic test is $1.71. This assumes a standard curve and a minimum of 20 specimens processed. We do not routinely perform the microbiological assay in our laboratory and are therefore unable to accurately assess its cost. However, in another investigator's laboratory (W. Hewitt, personal communication), the microbiological assay costs $0.75 per test, whereas a similar enzymatic assay costs $1.45 per test, considering reagents and wages.

The high degree of correlation obtained between the enzymatic and microbiological methods for gentamicin quantitation suggests that the enzymatic method can be used for routine serum analysis. The rapidity, accuracy, and sensitivity of the enzymatic method will allow for daily adjustments of patient dosage as the patients physiological status changes.

**APPENDIX**

The correlation coefficient, $r$, was calculated from the relationship:

$$r_{xy} = \frac{n \cdot 2xy - (2x)(2y)}{\sqrt{(n \cdot 2x^2 - (2x)^2)(n \cdot 2y^2 - (2y)^2)}}$$

The 95% confidence limit for the population correlation coefficient $\rho$ was obtained from Table 15 in ref. 6.

The standard error of the estimate $\sigma_y$ when the value obtained with one method was used to predict that obtained with the other from the linear regression was calculated from the relationship:

$$\sigma_y = \sigma_x \sqrt{1 - r^2}$$

**ACKNOWLEDGMENT**

This investigation was supported in part by Public Health Service Grant AI 06362 from the National Institute of Allergy and Infectious Diseases.

**LITERATURE CITED**