The acetyltransferase enzyme method for the assay of serum gentamicin concentrations and a comparison with other methods

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SYNOPSIS  The adenylyltransferase/acetyltransferase methods of gentamicin assay have been evaluated for accuracy, speed, and cost. For a comparable cost of materials the latter method is more accurate than that using the adenylyltransferase enzyme. The acetyltransferase method is much quicker than the adenylyltransferase due to the shorter time necessary for radioactive counting. Sonication is an easier method of enzyme preparation than the previously used osmotic shock technique. The acetyltransferase method is reproducible and there was a very good correlation between it and a microbiological agar-plate diffusion method.

The introduction of enzymatic methods for the estimation of gentamicin concentrations present in patients’ sera samples may enable a more accurate and reliable service to be offered by hospital laboratories. The original method of Smith, Van Otto, and Smith (1972) measured gentamicin by the formation of (14C)-adenylylated-gentamicin using (14C)-adenosine triphosphate (ATP) in the presence of a specific adenylylation enzyme derived from a gentamicin-resistant Escherichia coli strain. This method has recently been evaluated in comparison with the urease method (Noone, Pattison, and Samson, 1971) and the agar diffusion plate method (Reeves, 1972). It was shown by Ten Krooden and Darrell (1974) that there was least variation within serum samples using the adenylyltransferase method. Phillips, Warren, and Smith (1974) demonstrated a good correlation between the adenylyltransferase and agar plate diffusion methods (correlation coefficient > 0.95), but there was a poorer correlation of the urease method with either the adenylylation or agar diffusion plate methods (correlation coefficients 0.8 and 0.83). The purpose of this paper is to evaluate the published adenylylation method for both absolute accuracy and cost and also to contrast this method with an acetyltransferase method (Haas and Davies, 1973) which gives scope for greater accuracy than any of the methods yet published.

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Materials

(14C)-Adenosine triphosphate, (3H)-ATP, and (14C)-acyl coenzyme A were all obtained from the Radiochemical Centre, Amersham. Acetyl co-enzyme A was obtained from Sigma Chemicals. Analar grade chemicals were used where possible. Radioactivity was measured in a Nuclear Enterprises scintillation spectrometer. The scintillator used was 4-66 g of 2-(4'-t-Butylphenyl)-5-(4''-biphenylyl)-1,3,4-oxadiazole (Butyl-PBD) per litre of a 2:1 (v/v) toluene-triton X-100 solution.

Enzyme Preparation

Both Esch. coli strains were grown at 30°C in Oxoid nutrient broth no. 2. The gentamicin adenylyltransferase enzyme was prepared from Esch. coli JR76 by the osmotic shock method described by Beneviste and Davies (1971). The acetyltransferase enzyme was prepared from Esch. coli R5/W777 both by osmotic shock and also by sonication.

To prepare the enzyme by sonication an overnight (16-hour) culture of Esch. coli R5/W777 was centrifuged at 14 000 g for five minutes; the sedimented cells were then washed twice in 30 mM NaCl, 10 mM Tris-Cl, pH 7.8. The pellet from the second wash was resuspended in 0.5 mM MgCl2 at 4°C (5 ml of 0.5 mM MgCl2 per 100 ml of original culture volume). This suspension was then sonicated for 20 seconds using a Dawe type 3057 A Soniprobe.
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set to give 4 amps current. The cellular debris was removed by centrifugation at 25,000 g for 20 minutes, then the supernatant was divided into 1.0 ml aliquots and stored at −20°C.

Enzyme Assays for Gentamicin

Basic Adenylyltransferase Method

The basic adenylyltransferase method was as described by Smith et al. (1972), although some volumes have been altered. Each assay tube consisted of: 25 μl of buffer containing 10 μmoles Tris-Cl, pH 8.1, 1.6 μmoles MgCl₂, and 2 μmoles of dithiothreitol; 50 μl of enzyme extract; 25 μl of labelled ATP (quantity and specific activity as indicated in the text); and 25 μl of gentamicin dissolved in pooled human serum. The adenylylation was initiated by adding the enzyme extract and then incubating the assay tubes for 20 minutes at 30°C.

Following this incubation 75 μl of the solution was pipetted onto a 2.5 × 2 cm rectangle of Whatman P-81 phosphocellulose paper. As shown in fig 1 these rectangles are still attached to each other by a backbone strip of P-81 paper so that they could be conveniently handled together during the washing procedure. A simple holder for the backbone of P-81 was constructed of two plastic strips 1.5 × 0.3 × 60 cm which were clamped together by bulldog clips. The washing vessel was a 2-litre graduated cylinder. By this technique it was possible to handle 40-50 rectangles of P-81 paper in one batch.

The washing procedure did not include a step at 70°C, as described by Beneviste and Davies (1971). It was found that pipetting samples onto the P-81 and then washing all the papers together achieved a satisfactory replication of results without the need for a 70°C step. The washing consisted of three 2-litre washes in 10 mM Tris-Cl, pH 7.5 spread over a period of 10 minutes. The papers were dried at 120°C for 15 minutes before being placed in the scintillation fluid.

Acetyltransferase Method

The acetyltransferase method was as described by Haas and Davies (1973). Each assay tube consisted of: 20 μl of gentamicin dissolved in pooled human serum; 10 μl of (14C)-acetyl coenzyme A solution containing 2-75 nmoles of (14C)-acetyl coenzyme A, specific activity 30 μCi/μmole; 25 μl of buffer containing 3.0 μmole of Na-citrate buffer, pH 5.7, 0.3 μmole MgCl₂, and 0.1 μmole of dithiothreitol. The acetylation was initiated by the addition of 25 μl of enzyme extract to the assay solution, which was then incubated for 15 minutes at 30°C. Following this incubation 70 μl of the solution was pipetted onto a 2.5 × 2 cm rectangle of P-81 paper. The washing and drying procedures were as described for the adenylylation technique.

Agar Diffusion Plate Assay for Gentamicin

The agar diffusion assays for gentamicin were performed using 25 cm square plates of Oxoid DST agar (100 ml) surface seeded with a Klebsiella strain (Reeves, 1972). Five gentamicin standards (1.25, 2.5, 5.0, 10.0, and 20.0 μg/ml) were each set up in triplicate along with triplicate replication of each of the samples. These were distributed randomly in either the 30 or 45 wells cut in each plate by filling the wells level with the agar surface. The incubation was at 37°C for 18 hours and then the zone sizes were read to three figures with a zone reader.

The results were derandomized using a Hewlett Packard 9830A desk top computer and the triplicate replications of each serum sample were averaged. From the average values of the standards a polynomial line of best fit was computed and this was used to calculate the gentamicin concentrations present in the serum samples. The method for calculating the line of best fit for the standards was as published by Bennett, Brodie, Benner, and Kirby (1966).

Results

Preparation of the Enzyme Extracts

Figure 2 illustrates the standard curves obtained using a sonicate and an osmotic shockate extract of the cells. The standard curve is linear after 15 minutes' incubation with the sonic extract, but even after 60 minutes' incubation there is still a considerable curvature on the osmotic shockate standard curve.

The low enzyme activity in the osmotic shockate

1Leebrook Instrument Company Limited.
Adenylylation

Table I

<table>
<thead>
<tr>
<th>Method</th>
<th>Cpm Due to Gentamicin at 10 μg Gentamicin/ml</th>
<th>Background cpm at 0 μg Gentamicin/ml</th>
<th>Specific Activity of ATP (μCi/μmole)</th>
<th>Amount of ATP/Assay (nmole)</th>
<th>Curies/Assay (nCi)</th>
<th>Radioisotope Cost per Assay Tube (pence)</th>
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<td>750</td>
<td>Acetyl Co A</td>
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</table>

Table I Comparison of the enzymatic methods of gentamicin assay for both cost and the number of counts available

1Cost based on Amersham (UK edition) 73/74 catalogue.

(8-14C) ATP = £31 per 50 μCi
(2-3H) ATP = £30 per 1 mCi
(1-14C) acetyl CoA = £70 per 50 μCi.

is almost certainly due to the use of a 16-hour culture rather than one at the end of the exponential growth phase. However, sonication of the cells gave an adequate yield of the enzyme without the need of rigidly controlled growth conditions and the more complicated washing of the *Esch. coli* cells necessary for the osmotic shock procedure.

**STANDARD CURVES**
The range of counts obtained from various adenylyltransferase and acetyltransferase experiments is illustrated in figure 3. This shows the standard curve obtained for both (14C) and (3H)-ATP in the adenylation method and also that obtained using (14C)-acetyl coenzyme A in the acetylation procedure. A gentamicin concentration of 20 μg/ml serum gave 1200 and 1600 cpm for the adenylylase methods while the acetylation method gave 19 500 cpm.

**COST OF THE ASSAY**
Table I shows the cost of radioisotope used in each assay tube. This is not the total cost of a gentamicin determination; to obtain this figure a further 4-6p per assay tube must be added for the 10 ml of scintillation fluid that is necessary to count each 2.5 x 2 cm square of P-81 paper.

The original method of Smith *et al* (1972) is the most expensive, approximately £3-60 per clinical determination of gentamicin (five standards in duplicate plus duplicates of the specimen). Using tritiated ATP this cost falls to approximately £1-20 per specimen. With (14C)-acetoyl coenzyme A the cost per specimen is £2. The cost per specimen falls proportionally with each additional specimen in a batch since the greater part of the reagent costs with a single specimen is in the standards.

**ACCURACY OF THE METHOD**
Figure 4 illustrates the relationship between the microbiological and acetylation methods for the determination of gentamicin concentrations. Thirty-
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Fig 3 Standard curves for gentamicin assay. ■ Acetyltransferase assay with \(^{14}\text{C}\)-acetyl coenzyme A as described in the text. ● \(\text{(}^{3}\text{H})\text{-ATP adenyllyltransferase assay, }\text{V (}^{14}\text{C})\text{-ATP adenyllyltransferase assay, both the adenylation assays as described in the text and in table I.}

Fig 4 Double assay for gentamicin in 34 clinical serum samples by the agar plate diffusion and acetyltransferase methods. A calculated line of best fit as shown has a slope 1.07 and an intercept on the Y axis of -0.026.

four clinical serum samples were assayed for the gentamicin concentration by the microbiological plate diffusion method and then serum was stored at \(-20\text{°C}\). These stored serum samples were assayed by the acetylation technique. A correlation coefficient of 0.959 was found between the two methods. The theoretical line of best fit had a slope of 1.07 with an intercept of \(-0.026\).

The reproducibility of the acetylation method was assessed by repeated determinations of 2.5, 5.0, and 10.0 \(\mu\text{g/ml}\) gentamicin standards as samples. Table II gives the values obtained for 15 determinations of each sample and also compares these values with

<table>
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<tr>
<th>Method</th>
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<td>C.R.M.</td>
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<td>0</td>
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<tr>
<td>Mean % error</td>
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<tr>
<td>Standard deviation of % errors</td>
<td>5.49</td>
</tr>
<tr>
<td>Mean</td>
<td>8% error + 2 standard deviations</td>
</tr>
</tbody>
</table>

Table II Comparative accuracy of the enzymatic methods of gentamicin assay
those given by Ten Krooden and Darrell (1974) using the adenylyltransferase method. The results were assessed by calculating the mean percentage error for each set of samples and also the standard deviation of the percentage errors within that set of standards. To judge both skew and the spread of that skew in the results the figure used for assessing the accuracy was the mean percentage error value plus twice the standard deviation of these percentage errors in each set of results. This is the statistical method of analysis used in the national survey on gentamicin assay quality control (Reeves, 1975).

**TIME NECESSARY FOR THE ASSAY**

Ten Krooden and Darrell (1974) have estimated the time necessary for the adenylylation assay as 190 minutes. Over 60% of this time is occupied by counting the sample vials in the liquid scintillation spectrometer. They state that, providing all the reagents are from the same preparation, a single standard curve can be used repeatedly following a correction for the counts in the control serum. Although the acetyltransferase standard curves have been very similar for assays performed in these circumstances they are not sufficiently reproducible for this procedure to be recommended.

However, even by including a full set of standards in each assay, the time taken for the acetylation method is still less than that for the adenylylation due to the lower counting time of each sample. This easily enables an eight-hourly dosage regime of gentamicin to be adequately monitored.

**Discussion**

The methods so far published for the enzymatic determination of gentamicin concentrations have concentrated on the adenylylation method in which \(^{(14C)}\)-adenosine monophosphate is transferred from \(^{(14C)}\)-adenosine triphosphate to the gentamicin in the presence of a specific enzyme. The method has been validated with regard to both the agar plate diffusion method and the urease methods of assay. However, in accuracy versus speed it is limited by the low number of counts which are available for the assay. This is due to the relatively low affinity of the adenylylation enzyme for ATP and thus, in order to limit the cost of the radioisotope for the assay, only a low specific activity of ATP (1 or 5 \(\mu\)Ci/\(\mu\)mole) is available for the assay.

The acetylation method, in which gentamicin is labelled with a \(^{(14C)}\)-acetyl group from \(^{(14C)}\)-acetyl coenzyme A in the presence of an acetyltransferase enzyme, offers a much greater number of counts, due to the high affinity of this enzyme for acetyl co-enzyme A. This enables a high specific activity (30 \(\mu\)Ci/\(\mu\)mole) substrate to be used for a comparable cost to the adenylylation assay.

Previous methods for preparing the enzyme extracts have used an osmotic shockate of an *Esch. coli* culture at the end of the exponential growth phase. This requires a growth time of eight to 10 hours which is difficult to fit to a routine laboratory preparation. Therefore sonication, as described earlier, was routinely used as a means of preparing the enzyme from an overnight (16-hour) culture.

A clinically realistic serum gentamicin concentration by which to judge the efficiency of the adenylylation and acetylation methods of assay is 10 \(\mu\)g/ml. Table I demonstrates that using the adenylylation method, except in the results of Furger, Russi, and Kayser (1973), the ratio of counts at 10 \(\mu\)g/ml to the background with 0 \(\mu\)g gentamicin/ml is 4-5. However, with the acetylation method this ratio is over 12. As the gentamicin concentration decreases, this ratio will be even smaller. The low sample count rate to background count rate for the adenyltransferase method will further increase the counting error, especially when lower gentamicin concentrations are being measured. Table II demonstrates that for both the acetylase and adenyrase methods there is a decrease in reproducibility at the lower gentamicin concentrations, but this is much more pronounced with the published adenyrase results. The published results of Phillips et al (1974) do not contain sufficient data to allow this analysis to be performed on their assays. To estimate the reproducibility of their results Phillips et al (1974) calculated the coefficient of variation (%) which was 5-1% for the adenylylation method. Calculation of the coefficient of variation for the 2-5, 5-0, and 10-0 \(\mu\)g/ml standards in the acetylation method (table II) gave values of 5-5, 4-8, and 4-6%. Although the accuracy of the two methods appears to be comparable by this statistical method the overall counting time for the adenylylation method of Phillips et al (1974) must be longer than the acetylation method published here, since, as shown in table I, there is a very low level of counts (200-250 cpm) at 10 \(\mu\)g/ml which was only four times the background rate. To achieve the reproducibility as shown in their coefficient of variation and also in the lack of variation in their standard curve, as shown in fig 1 of their paper (Phillips et al, 1974), the counting times for each sample must have been at least 10-20 minutes.

The number of counts determines the length of time each sample must be counted in order to reduce the relative standard deviation of the counting error to 1%. Ten thousand counts have to be collected to achieve this accuracy. For the adenyrase methods a 10 \(\mu\)g/ml sample has to be counted for at least
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10 minutes, whereas a counting time of one minute was adequate for the acetylation method.

On the basis of published results the acetylation method is apparently more accurate than the adenylylation method for a comparable cost of materials. This cost is not prohibitive (£2 for a single clinical specimen), although this is more than the cost by the standard microbiological agar plate diffusion method. However, as the national survey on gentamicin assay quality control has disclosed (Reeves, 1975), microbiological methods leave much to be desired when used in many laboratories, perhaps because they are open to error at many stages. The acetylation method may be more accurate for routine use, as it can be broken down into a number of distinct, well defined operations. The results presented here with the acetylase method are a significant improvement over those published for the adenylase method and are also better than the most accurate results obtained by microbiological techniques submitted to the survey of gentamicin assay quality control.

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