A SPECTROPHOTOMETRIC ASSAY FOR GENTAMICIN*

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A rapid and accurate spectrophotometric assay has been developed for the determination of blood serum levels of gentamicin and related antibiotics. The assay uses a purified enzyme from *Escherichia coli* JR88/C600 that acetylates gentamicin with the production of coenzyme A, linked to a chemical reaction with a sulfhydryl reagent to produce stoichiometric amounts of a sensitive chromophore, monitored in the visible spectrum. The system provides advantages of speed, cost, convenience, accuracy, and enzyme stability to the desirable characteristics encountered with previous enzymatic methods.

It is becoming increasingly apparent that safe and effective use of many drugs is dependent upon a monitoring of blood serum levels of the drug in patients undergoing drug therapy. A need for determinations of blood serum levels of gentamicin clearly exists since a minimum concentration of 4 μg/ml is recommended for therapeutic effectiveness, toxicity is encountered when serum levels exceed 12 μg/ml, and the response of individual patients to a given dosage regimen variesw idely[1,2]. Several assay methods have been developed for clinical use, the most recent being an enzymatic method employing a radioactive tracer[3,4,5]. SMITH *et al.*[5,6] have clearly shown that in addition to other advantages such as convenience and specificity, the enzymatic assay provides greater accuracy and precision than a comparable microbiological assay. This report describes an alternative enzymatic assay which adds the economy, convenience, and greater reliability of a spectrophotometric detection method to the advantages of the earlier enzymatic method.

Materials and Methods

Purified gentamicin C_{1a} was provided by Dr. GERALD WAGMAN of Schering Corporation, Bloomfield, N.J. The preparation was estimated to be 93~95% pure on the basis of chromatographic analysis. Human serum was obtained from the University Hospitals of the University of Wisconsin. Tris (hydroxymethyl) aminomethane (Tris), ethylenediamine tetracetic acid (EDTA), and 5, 5′-dithiobis-(2, 2) nitrobenzoic acid (DTNB) were purchased from Sigma. Acetyl coenzyme A (Acetyl CoA) was from P-L Biochemicals, and ammonium sulfate (enzyme grade) was from Schwarz-Mann. Gentamicin acetyl transferase I (GAcT I) first isolated from *P. aeruginosa* 130[7], was purified from R-factor containing *E. coli* JR88/C600, according to the method of WILLIAMS and NORTHROP[8]. The regression analysis of the data was done by the Madison Academic Computing Center Program REGAN 2.

The procedure for the spectrophotometric assay of gentamicin is a modification of the

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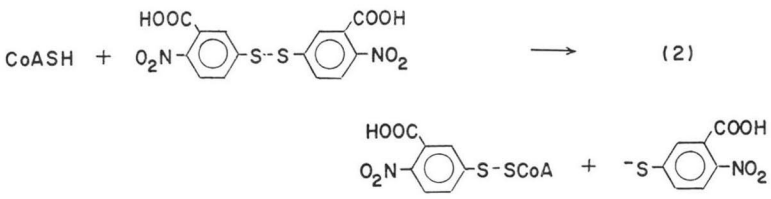
method of BENVENISTE and DAVIES[9], developed for kanamycin acetyl transferase. GAcT I catalyzes reaction 1 to produce a stoichiometric amount of gentamicin acetylated in the 3 amino position and a free sulfhydryl group in the form of coenzyme A[7].

$$\text{Gentamicin} + \text{Acetyl-CoA} \rightarrow \text{Acetyl-Gentamicin} + \text{CoASH} \quad (1)$$

Sulfhydryl groups rapidly react with DTNB to produce a stoichiometric amount of a disulfide and thionitrobenzoic acid, as shown in reaction 2[10].



The components of reaction 2 do not effect the progress of reaction 1[8], hence the two reactions can be conducted simultaneously in a single-step procedure. The reactions were monitored by recording the increase in absorbance at 412 nm due to the production of thionitrobenzoic acid, using a Gilford model 240 spectrophotometer coupled to a Leeds and Northrup recorder equipped with variable scale deflections and a multispeed chart drive. Full scale optical densities of 0.4 and 1.0 and a chart speed of 1 inch per minute were used. The temperature of the cuvette compartment was maintained at 25°C.

Assays of gentamicin were carried out at pH 7.8 in cuvettes having a 1-cm light path and a total reaction volume of 2.5 ml containing 5 mM Tris-HCl, 0.1 mM EDTA, 5 mM $(NH_4)_2SO_4$, 70 μM acetyl CoA, 250 μM DTNB, 0.005 international units of GAcT I, and 1 ml of serum containing gentamicin. Samples of serum were prepared by adding microgram amounts of gentamicin to 1 ml of normal serum and incubating the mixture at 25°C for 10 minutes. Deionized water was substituted for serum in assays done in the absence of serum. Reactions were started by the addition of acetyl CoA and DTNB. Control assays lacking gentamicin were conducted with each determination and found to give results similar to those obtained in the absence of enzyme. Initial attempts to apply the spectrophotometric assay were unsuccessful prior to the development of a purification method for the enzyme. Protein impurities present in crude extracts react with DTNB to produce background rates greatly in excess of the enzymatic rate. Consequently, a partially purified preparation of GAcT I is a minimal requirement for the assay, and a highly purified preparation is necessary for maximum precision.

Results

Assays conducted in the absence of serum were complete in less than 5 minutes, and the absorbance of thionitrobenzoate at 412 nm was stable for at least 15 minutes. Fig. 1 shows the dependence of the absorbance upon the amount of gentamicin C_{1a} present. The plot is linear from 0 to 50 μg of gentamicin, with a slope of 0.0116 ± 0.00008 O.D./μg.

In contrast, assays conducted in the presence of serum required more than 15 minutes to reach completion, and the absorbance at 412 nm continued to increase after the added gentamicin was depleted. Fig. 2 shows the time course of the reactions in the presence and absence of serum, and illustrates both the decreased rate of development of gentamicin-dependent absorbance and the background absorbance. Nevertheless, Fig. 3 shows that the gentamicin-dependent absorbance in the presence of serum is also linear from 0 to 50 μg of gentamicin, with a slope of 0.00837 ± 0.00011 O.D./μg. The data of Fig. 3 was obtained by subtracting the

Fig. 1. The relationship between the absorbance of thionitrobenzoate and gentamicin in the spectrophotometric assay.

Assays were performed in duplicate in the absence of serum and read after 7 minutes of reaction time.

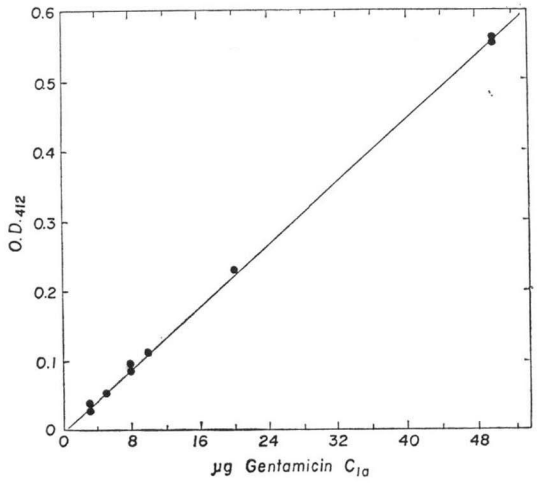


Fig. 2. The time course of the spectrophotometric assay in the presence and absence of serum, at different levels of gentamicin.

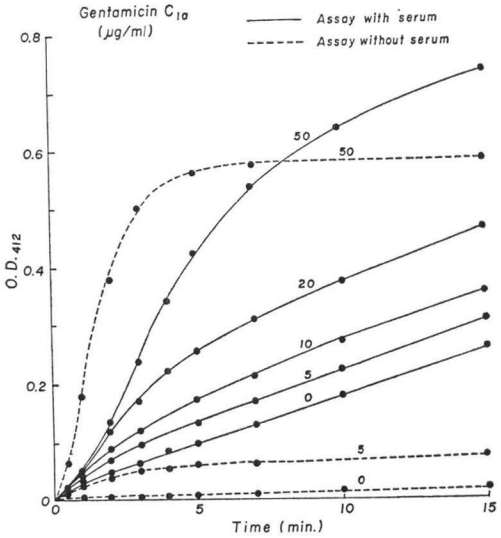
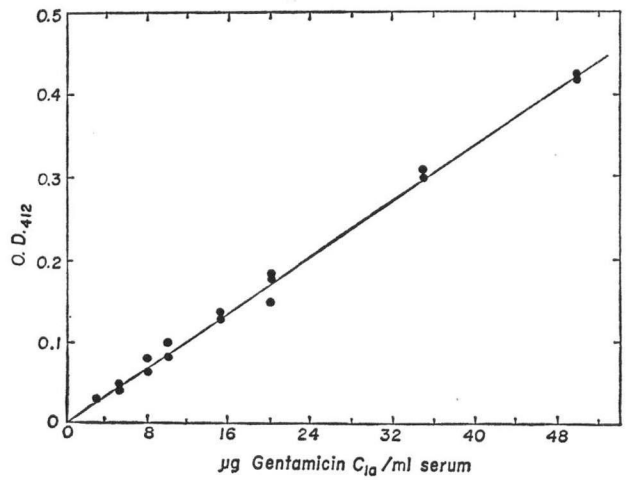


Fig. 3. The relationship between the absorbance of thionitrobenzoate and gentamicin in the spectrophotometric assay, conducted in the presence of serum.

Assays were performed in triplicate, read after 7 minutes of reaction time, and corrected for background reactions between serum and DTNB.



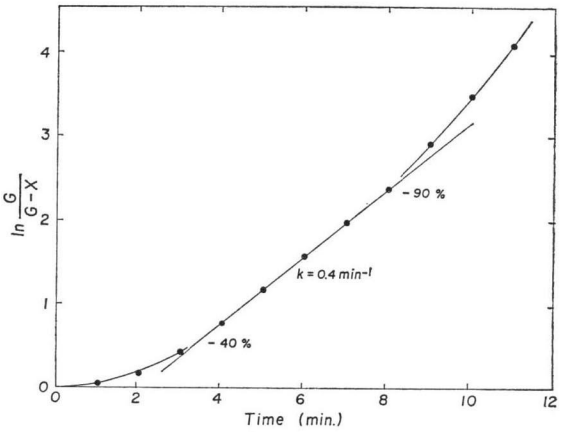
absorbance of control assays which were run simultaneously and contained no added gentamicin. Measurements were made at 7 minutes after the start of each reaction. Allowing the gentamicin reaction to go further towards completion increased the sensitivity of the assay, but decreased the precision obtained because of larger background corrections. For example, at 15 minutes a linear plot was obtained with a slope of 0.00942 ± 0.00018 O.D./μg.

The cause of the decreased rate of reaction in the presence of serum was further examined.

Increasing the amount of GAcT I or DTNB added to the assay did not significantly change the rate of the reaction. Fig. 4 shows a first order plot of the progress of one assay in serum, and reveals a linear fit of the data covering approximately half of the reaction progress. This finding indicates that a first order process with a rate constant of 0.4 min^{-1} is rate-limiting. The linear portion of the plot is preceeded by an initial lag, also seen in Fig. 2, which apparently reflects the necessity of forming a sufficient steady-state level of free coenzyme A to satisfy Reaction 2, since the lag is reduced by increasing the concentration of DTNB. The plot also deviates from linearity after approximately 8 minutes of reaction time. The reason for this latter deviation is uncertain. However, this portion of a first order plot is extremely sensitive to errors of measurement, which in this case are augmented due to higher background corrections.

Fig. 4. First order plot of the disappearance of gentamicin in the spectrophotometric assay in the presence of serum.

Data were taken from Fig. 2, representing the time course for the assay of 50 μg of gentamicin. The percentage markings indicate the amount of gentamicin that has undergone acetylation.



Background rates were found to be proportional to the amount of serum protein added, and apparently reflect a reaction of DTNB with free sulfhydryl groups of these proteins[10]. Blocking or oxidizing these sulfhydryls prior to conducting assays may provide a means of reducing background absorbencies.

Discussion

A comparison of the precision of the spectrophometric-enzymatic the radioisotopic-enzymatic, and a microbiological assay for gentamicin in the presence of serum is shown in Table 1. In the desired clinical range of concentrations of gentamicin (4～12 μg/ml of serum) the spectrophotometric assay is significantly more precise than the other methods. At lower concen-

Table 1. Comparison of enzymatic and microbiological assay*

Gentamicin (μg/ml)	SD (μg/ml)			% Expected error		
	E	E_{14C}**	M**	E	E_{14C}**	M**
0.1～ 5.0	0.262	0.049	0.609	8.7	1.9	23.9
5.0～10.0	0.74	2.24	4.20	9.9	23.9	52.0
10.0～15.0	0.78	1.85	4.85	6.3	14.8	38.8
15.0～30.0	1.37	13.63	26.03	6.1	60.6	115.7
30.0～50.0	0.82			1.7		

* SD=standard deviation; E=spectrophotometric assay using gentamicin acetyl transferase I (data derived from Fig. 3); E_{14C}=radioactive assay using gentamicin adenylyl transferase; M=microbiological assay

** Data from SMITH and SMITH[5].

trations, it is less precise than the radioactive method due to a difference in sensitivity limits. If the higher sensitivities obtainable by radioactive methods at low gentamicin concentrations are desired, GAcT I may still be employed using ^{14}C-acetyl CoA according to the assay procedures of HAAS and DAVIES[14] as developed for kanamycin acetyl transferase. This radioactivity assay is preferable to the adenylylating system because of the more favorable characteristics of the GAcT I enzyme. In addition, the sensitivity of the spectrophotometric assay at low concentrations of antibiotic can be increased either by using cuvettes with a longer path length (10 cm cells are currently employed in this laboratory for investigating the kinetic mechanism of GAcT I), or possibly by linking the enzymatic reaction to a sulfhydryl reagent having a larger extinction coefficient than DTNB (ε_{412}=15,700)[11] such as 4, 4′-dithiodipyridine (ε_{324}=19,800)[12] currently under investigation. The greatest difference between assays appears at higher concentrations of gentamicin. Both the radioisotopic and microbiological assays become unreliable once toxic levels of gentamicin are attained, whereas the percentage error of the spectrophotometric assay actually decreases as a function of gentamicin concentration. The accuracy of the three assay methods is similar when results are interpreted on a relative scale based upon known concentrations of antibiotic. However, the purity of antibiotic preparations does vary, and greater accuracy may be obtained with the spectrophotometric assay by reference to the extinction coefficient of DTNB as an internal standard[11]. In contrast, the radioactive method requires an external standardization in the form of a determination of the specific activity of ^{14}C-ATP, while the microbiological method can only be performed on a relative scale.

The spectrophotometric assay presents several other advantages. The convenience of a single-step procedure, adaptable to most visible absorption spectrophotometers, as opposed to the multiple requirements of scintillation counting of radioisotopes or agar diffusion measurements of microbial cultures is readily apparent. The present assay is also much faster than the alternatives. Results of gentamicin blood levels can be obtained in approximately 15 minutes *vs.* 2 hours for the radioisotopic and upwards to 24 hours for the microbiological assays[6]. The time required by the assay is very important if dosage schedules are to be adjusted to achieve optimal therapeutic levels yet avoid toxicity. Less obvious are advantages associated with the acetylating enzyme. GAcT I has been purified to homogeneity in high yield (one liter of R-factor *E. coli* broth yields 2 units of pure enzyme, specific activity=4) and found to be stable to lyophilization and long term storage[8]. Approximately 10% of the enzymatic activity is lost by lyophilization, but no further loss during storage for several months has been detected. In contrast, the adenylating enzyme is recovered in low yield by partial purification (one liter of R-factor *E. coli* broth yields 4×10^{-4} units of enzyme, specific activity $=3\times10^{-5}$) and is highly unstable, requiring elaborate storage procedures[5]. Finally, an economic savings of at least 50% per assay is achieved since the high cost of radioisotopes is avoided. The only apparent disadvantage to the spectrophotometric assay is the volume of serum required. However, the assay has been performed in microcuvettes using 0.1 ml of serum instead of the 1 ml as described, with no loss of sensitivity.

The broad specificity of GAcT I provides the opportunity of applying the spectrophotometric assay to the determination of blood levels of other antibiotics, including sisomicin, kanamycin B, and tobramycin[7]. In addition, the principle is applicable to clinical assays using other acetylating enzymes, notably kanamycin acetyl transferase[9], to extend the range of aminoglycoside antibiotics subject to serum monitoring. However, this enzyme has yet to be purified. The possibilities of discovering and purifying other aminoglycoside acetylating enzymes should be encouraged, since it may be possible to assay all aminoglycoside antibiotics by this method.

The effect of serum on the time course of the assay, shown in Fig. 2, together with the apparent first order kinetic behavior of the effect shown in Fig. 4, suggests an additional and important application of the spectrophotometric assay to the field of pharmacokinetics. The simplest interpretation of these observations is that the reduced rate of reaction with GAcT I is due to binding of gentamicin to serum proteins. Thus, once unbound gentamicin is con-

sumed by GAcT I, the measured reaction becomes dependent upon the rate of dissociation of bound gentamicin from the serum protein complex. The process should be first order and independent of enzyme and DTNB concentrations as observed, however further studies will be necessary to establish the hypothesis. Serum binding of gentamicin is known to occur, at a level of approximately 30%[13], but varies with individual serum samples and is currently measured by equilibrium methods. By providing a measure of the rates of dissociation of antibiotics from serum protein, the spectrophotometric assay may prove to be useful as a rapid means of determining individual variations in serum binding and, more importantly, add a dynamic dimension to the effect of serum binding on pharmacokinetic parameters such as bioavailability and rates of distribution and excretion. Such parameters are dynamic functions, and pharmacokinetic calculations are poorly served by equilibrium binding measurements, which are static functions. For example, the mean circulation time of blood in the capillaries of the vasa recta of the kidney medulla is 50 seconds[15], while the data of Fig. 4 indicates a half-life of 1.7 minutes for the gentamicin-serum protein complex. Hence, by combining these values in a first order kinetic calculation, it can be estimated that 28% of the bound gentamicin may be released from serum protein and excreted in urine. Such an estimate can not be made from equilibrium binding data.

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