Non-equilibrium Enzyme Immunoassay of Gentamicin

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We describe a fast, accurate non-equilibrium enzyme immunoassay for serum gentamicin. With use of isokinetic conditions an assay takes a total of 12 min, including regeneration of the immunosorbent. The coefficient of correlation between the present method and conventional microbiological assays was 0.98.

Additional Keyphrases: calorimetry, TELISA (Thermometric Enzyme-Linked Immunosorbent Assay), analytical systems, drug assay, enzyme thermistor

The ototoxic effect of gentamicin is well established. Because the therapeutic and toxic concentrations of gentamicin in serum are so near to one another, serial monitoring of gentamicin is the most reliable therapeutic guide. This paper describes a rapid, specific, and accurate enzyme immunoassay method.

Enzyme immunoassay has some inherent advantages over radioimmunoassay (1). Conventional immunoassays, apart from the homogeneous enzyme immunoassay (EMIT, Syva) (2) and a kinetic method based on the combination of solid-phase technique and continuous flow (3, 4), take at least a few hours.

We have earlier described the use of a simple calorimeter for following the course of the enzyme reaction caused by the marker enzyme on the antigens used in the enzyme immunoassay (3, 5). This simple flow-calorimeter, called the "enzyme thermistor," has earlier been applied in conventional clinical analysis (6, 7) and environmental analysis (8, 9), as well as in process control (10).

In the Thermometric Enzyme-Linked Immunosorbent Assay (TELISA), a microcolumn filled with a polymer support containing covalently bound antibodies has been used. This microcolumn is well insulated from the surroundings. A continuous flow is pumped through the antibody bed. Before the antigen-containing sample to be analyzed is introduced, a fixed amount of enzyme-labeled antigen is added. On passage of the sample through the immunosorbent in the enzyme thermistor column, competitive binding of native and enzyme labeled antigen to the antibodies will take place. As there is a continuous flow, no separate washing steps are needed. The amount of enzyme bound to the column is measured by introducing a pulse of substrate. As the substrate is converted into product, heat is evolved and measured. The change in temperature of the solute passing by the heat sensitive semiconductor induces a change in resistance. This change is amplified and registered on a strip chart recorder. The amount of enzyme bound is a function of the concentration of native antigen in the sample. With use of standard curves it has been possible to assay, e.g., human serum albumin down to $10^{-13}$ mol/liter (5).

This paper reports the use of this system in analysis of a hapten of clinical interest, gentamicin. The choice of the substance to be assayed was partly influenced by the interest in a hapten assay. This, in combination with a molecule of clinical interest, decided the choice. Several methods for determining gentamicin are available (11-17), but the fastest is a rather laborious technique involving extraction, derivatization, and separation with high-performance liquid chromatography (18).

Materials and Methods

Hydrogen peroxide (300 g/liter) was obtained from BDH Ltd. Poole, U.K. Cyanogen bromide was purchased from Fluka AG, Buchs, Switzerland. Catalase, type C-30 from beef liver, N-hydroxy-succinimide, and 1-ethyl-3-(3-dimethylaminopropyl)-carboimide were all from Sigma Chemical Co., St. Louis, Mo. 63178. Sepharose CL-4B and Sephadex G-200 were from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Gentamicin sulfate was a generous gift from Schering Corp., Kenilworth, N.J. 07033, and from Merck, Darmstadt, West Germany.

[3H]Gentamicin sulfate (637 Ci/mol, 1.0 Ci/liter) was obtained from the Radiochemical Centre, Amersham, U.K. All other chemicals used were of analytical grade. Control serum (lyophilized) was obtained from Dade, Miami, Fla. 33152. Antiserum against gentamicin was raised in rabbits by immunizing with a hapten coupled to a carrier, and with use of Freund's complete adjuvant. Bleedings were started two weeks after the second immunization, twice a week for three weeks. All serum samples obtained were stored at $-20\,^\circ\text{C}$ until used (19).

Apparatus

The spectrophotometer used was a Beckman UV 5230. The enzyme thermistor unit developed at the Chemical Center, Biochemistry 2, University of Lund, consists of the following components: 1) Wheatstone bridge, Knauer Temperature Messgerät, Wissenschaftlicher Geraetbau, West Berlin, Germany. 2) Enzyme Thermistor Unit with a thermistor type VECO 41 A 28. 3) Pump, Varioi perpex LKB, Bromma, Sweden. 4) Water-bath, Hetotherm Model 06 PG 623 UO, Heto, Birkerod, Denmark. The liquid scintillation counting system was a Mark III Scintillation System, Model 6880 (Searle Analytic Inc., Des Plaines, Ill. 60018).

Procedures

Purification of anti-gentamicin antibodies. Anti-genta- micin antibody was purified by immuno-affinity chroma-
Gentamicin was immobilized onto Sepharose CL-4B by BrCN-activation (20). The polysaccharide was activated for 8 min at pH 10.7 with 86 mg of BrCN per gram of moist gel. After washing with cold 0.1 mol/liter NaHCO₃, coupling was performed during 15 h at 4°C with 6 mg of gentamicin sulfate per gram of moist gel. After a wash with cold 0.1 mol/liter NaHCO₃, the gel was packed into a column (Whatman, 200 × 10 mm) and equilibrated with potassium phosphate buffer (0.1 mol/liter, pH 7.0) at a flow rate of 7.5 ml/h. One milliliter of rabbit antiserum was applied to the column. Washing with phosphate buffer was continued until the eluate was optically clear at 280 nm. The anti-gentamicin was eluted with 0.2 mol/liter glycine-HCl, pH 2.2, by using a reversed flow through the column (21). We collected 1.5-mI fractions containing antibodies in test tubes prefilled with 0.5 ml of 0.25 mol/liter potassium phosphate, pH 7.5, pooled them, and dialyzed this pool against 0.1 mol/liter potassium phosphate buffer, pH 7.0. The antibody was then concentrated to 1 g/liter with the aid of collodion bags.

Immobilization of anti-gentamicin antibody on agarose (Sepharose). Antibodies were immobilized on Sepharose CL-4B by using gel activated with 70 mg BrCN per gram of moist gel. After activation for 8 min at pH 10.7, coupling (0.2 mg of antibody added per gram of gel) in 0.1 mol/liter NaHCO₃ was allowed to continue overnight.

Preparation of gentamicin-catalase conjugates. Carboxylic groups in catalase (EC 1.11.1.6) and amino groups in gentamicin were coupled by pre-modifying the carboxyl groups into active esters by means of a water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] and N-hydroxysuccinimide (22).

To 1.0 ml of cold phosphate buffer, pH 6.5, was added 3.2 mg of N-hydroxy-succinimide and 2.8 mg of water-soluble carbodiimide, and then 500 μl of catalase (23 g/liter). The activation of catalase was allowed to continue for 15 min before 25 μl of [3H]gentamicin (39 nmol) and 2.5 mg (4.6 μmol) of gentamicin were added. The reaction continued overnight, after which 0.5 ml of phosphate-buffered 1.5 mol/liter glycine, pH 7.0, was added. The mixture was dialyzed against potassium phosphate (0.1 mol/liter pH 7.0) and separated by gel chromatography on Sephadex G-200 (900 × 15 mm column).

The fractions containing protein were tested for catalase activity (23) and the amount of gentamicin was calculated from the radioactivity of the conjugates. The protein concentration was 0.9 μmol/liter and the gentamicin concentration was 0.7 μmol/liter. The total yield of the reaction as a relatively homogeneous, soluble conjugate was estimated to be approximately 1%, as judged from the enzyme content.

Assay procedure. In the enzyme thermistor unit used, antibodies covalently bound to Sepharose CL-4B were packed into a 1.0-ml column. A constant amount of gentamicin-catalase conjugate was mixed with the sample containing an unknown amount of free gentamicin. The mixture was pumped through the column at a flow rate of 0.7 ml/min. The sample pulse was followed by washing with buffer and 1.0 mol/liter KCl. During the short exposure, competitive binding of gentamicin and gentamicin-catalase took place on the antibody column. To eliminate disturbing effects from non-specific protein adsorption onto tubing and column material, we used the short pulse of KCl.

After subsequent administration of hydrogen peroxide, the heat of the enzyme reaction was recorded as the change in the resistance of the thermistor. The height of the signal thus obtained was inversely proportional to the amount of gentamicin in the sample.

A typical example of a gentamicin assay would be as follows: Add 50 μl of the gentamicin-catalase conjugate to a solution containing 100 μl of serum and 850 μl of potassium phosphate buffer, pH 7.0. Introduce a 1-min pulse (flow rate, 0.7 ml/min) of this mixture into the flow, followed by a 1-min pulse of 1.0 mol/liter KCl. Then administer a 90-s pulse of 2 mmol/liter solution of hydrogen peroxide to the system. After recording the heat of the reaction, wash the column for 2 min with a glycine-HCl buffer (0.2 mol/liter, pH 2.2), to dissociate the antigen–antibody complex. The column is then ready for use in a new assay.

Results and Discussion

A diagram of the assay cycle is given in Figure 1. It is seen that after addition of the antigen-containing sample, a pulse of high salt concentration is necessary to eliminate the effects of all nonspecifically bound protein. The heat of the reaction between the marker enzyme and its substrate is then recorded. The assay cycle is terminated by a pulse of glycine-HCl (0.2
mol/liter, pH 2.2), to wash off all bound antigen before a new sample can be introduced.

Standard solutions of gentamicin dissolved in buffer as well as in the control serum (Dade) were analyzed and standard curves were obtained (Figure 2). The shape of the standard curves could be changed by varying the amount of antibody in the column (5). A concentration range of 10 to 400 μg/liter was used for routine assays, i.e., serum samples with therapeutic concentrations of gentamicin had to be diluted before assay.

The antigen–enzyme conjugate obtained was prepared by coupling gentamicin to 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide- and N-hydroxysuccinimide-activated carboxyl groups on the protein.

A gel-filtration step was included (Sephadex G-200) to separate unreacted gentamicin not removed in the dialysis step from enzyme-gentamicin conjugate. A typical elution profile is shown in Figure 3. 3H-labeled hapten was used to demonstrate the presence of gentamicin, and, as can be seen in the figure, there is a fair amount of "high-molecular" gentamicin. It was not possible, however, to separate unreacted enzyme from the complex, but since nothing suggested that nonspecific binding of free enzyme disturbed the subsequent analyses, we made no efforts to separate such enzyme. If such problems should arise, an affinity step based on specific interaction with the other moiety of the complex (here gentamicin) must be applied (24, 25). The complex obtained was stored in potassium phosphate buffer (0.1 mol/liter, pH 7.0) at 4 °C. No decrease in either activity was observed during storage for three months.

The within-day coefficient of variation was 2% in the therapeutic interval (3–5 mg/liter). Day-to-day variation is shown in Table 1.

An interesting point when assaying such a small molecule as gentamicin is the specificity of the antibodies, even though the probability of an unexpected presence of other similar antibiotics in serum samples to be analyzed is negligible. Tobramycin and kanamycin, both aminoglycoside antibiotics very similar in structure to gentamicin, were tested, but as is seen in Table 2 no interference was demonstrable, even for concentrations far exceeding therapeutic concentrations.

The concentration values of gentamicin from serum samples obtained with the TELISA-technique were compared with those obtained with the conventional microbiological technique (26), giving values accurate to 0.5 mg/liter. Further, concentrations less than 1 mg/liter could be measured only as "<1 mg/liter." In the comparison made here, all these low values are given as 0.5 mg/liter. As can be seen from Figure 4, results obtained by the two methods correlated well (r = 0.98), and the intercept of the ordinate was 0.002.

Analytical recovery of pre-determined concentrations of gentamicin was 100–110%. The analyses were carried out in concentrations up to 0.4 mg/liter, where the standard curve is still steep and the accuracy high.

The general scheme given in Figure 1 indicates that an assay, including regeneration of the system, requires a total of 12 min. This time is equal to or shorter than that required by other methods—although we are aware that speed is not a critical factor in this assay, because gentamicin is not administered by sustained intravenous infusion, nor is its biological half-life extremely short.

As discussed in detail elsewhere (5), the assay cycle includes some critical steps. First, binding of antigens to the immobilized antibodies. Second, washing off of the antigens—native as well as labeled. The exposure of the antibodies to the antigens in the flow is brief (1–2 min); consequently the system is far from equilibrium. Instead, this procedure is characterized by a short, well-defined, and highly reproducible time of

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### Table 1. Precision of the Assay

<table>
<thead>
<tr>
<th>Gentamicin concn., mg/liter</th>
<th>Found, mg/liter</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>2.0</td>
<td>2.1</td>
<td>5.1</td>
</tr>
<tr>
<td>4.0</td>
<td>4.1</td>
<td>3.0</td>
</tr>
<tr>
<td>8.0</td>
<td>7.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*The sample was a serum containing gentamicin, divided into small portions, frozen, and stored in a deep freeze.*

*Mean of five to seven determinations made during eight days. All values were calculated from the same standard curve used the first day.*

### Table 2. Effect of Some Other Aminoglycosidic Antibiotics

<table>
<thead>
<tr>
<th>Substance</th>
<th>Conc. applied, mg/liter</th>
<th>Change in response, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>21.3</td>
<td>-3</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>2.5</td>
<td>+7</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>16.0</td>
<td>0</td>
</tr>
</tbody>
</table>

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**Fig. 4.** Serum gentamicin, as measured by the present method vs. the conventional microbiological technique

Coefficient of correlation 0.98
contact. Therefore, only a fraction of the antigen molecules in the sample is bound to the sorbent, but a very reproducible fraction. In experiments with successive assays of identical samples, variation has been less than 2%. This high reproducibility is due to the few pipetting steps and the well-defined conditions for contact between antigens and antibodies. Thus, even though the exposure is brief, the results so far obtained are fully satisfactory. The other step that seems critical is the regeneration of the immunosorbent, i.e., the whole principle of re-using the sorbent. It was, however, shown that the dissociation of the complex by glycine-HCl buffer (0.2 mol/liter, pH 2.2) left no enzyme activity on the column. It deserves mentioning that the antibodies used in these studies were purified by immunosorption techniques. In the subsequent desorption step, this same glycine buffer was used routinely. This means that if initially there were fractions of antibodies with a very high avidity for the antigen, they might adhere to the immunoaffinity column, with the result that all antibodies applied for immobilization are those that responded favorably to the eluting procedures used. It was not possible to demonstrate that these properties of the antibodies changed upon immobilization. Antibodies may be denatured, as judged from a slow day-to-day decrease in capacity of the column, observed as an increase in steepness of the initial part of the standard curve. However, a fresh column was characterized by the same standard curve for several days, after which the decrease was very slow, so that changes in the standard curve were at most a few per cent a day.

Work is now in progress concerning reversible immobilization (27, 28) of the antibody so that also antibodies with a very high affinity (29, 30) for their respective antigens may be used in the assay.

It should be mentioned that the assay described here can be used also in combination with other transducers, e.g., specific electrodes (4) or spectrophotometers.3

In its present stage of development, the method is easily accessible, rapid, and can be used to assay one to 25 samples a day.

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References