Improved Approach to Sequential Addition Immunoassay

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In the usual sequential addition enzyme immunoassays for drugs, the activity of the drug-labeled enzyme decreases continuously with time as more of it is bound to antibody. Sensitivity also decreases; the activity immediately after mixing is the most sensitive indicator of drug concentration. The reaction of enzyme-drug with antibody can be stopped by saturating the antibody with a larger quantity of unlabeled drug, which reacts with the antibody faster than does the enzyme-labeled drug. When drug is added soon after the reaction starts, the enzyme activity is stabilized and the sensitivity to small quantities of antigen is increased. This approach, with modification, should be applicable to sequential immunoassays in which other kinds of labels are used. The enzyme activity can be measured for a longer time, with the predictable increase in precision, as well as the ability to detect smaller quantities, to use less reagent, and to use end-point rather than kinetic assays.

In the EMIT (Enzyme Multiplied Immunoassay Technique; Syva Corp., Palo Alto, Calif. 94304) for drugs in serum (1), the serum is mixed with a solution of an antibody to the drug. A solution of the same drug covalently coupled to an enzyme not found in serum is then added and the activity of the enzyme is measured.

The enzyme-coupled drug binds to the antibody on binding sites not occupied by drug. In so doing, the enzyme activity is changed—in this instance diminished. The remaining enzyme activity is thus a function of the concentration of the drug in the serum. The concentration is determined by comparing the enzyme activity with that produced by standard solutions of the same drug.

We previously reported studies of the EMIT reactions by use of the Oak Ridge National Laboratories miniature Centrifugal Fast Analyzer (2). We noted that the rate of each enzyme-catalyzed reaction decreased continuously with time. With different concentrations of free drug, the rates, although initially different, tended to decrease at different rates and to converge on a common value.

We interpreted the decrease as indicative of a continuing reaction of enzyme-labeled drug with antibody. The concentration of drug was most sensitively indicated by the enzyme activity immediately after the reagents were mixed. We therefore postulated that we could increase the sensitivity of the assay if we could limit the reaction to those antibody sites that reacted first. We therefore used the activity measured from 15 to 30 s after the reagents were mixed as the measure of the drug concentration. This was the soonest and the briefest interval over which we could measure the rate with acceptable precision.

Since the EMIT procedure requires measurement of activity over only a short time, it seemed to be making less than full use of the potential of an enzyme as a label for immunoassay. If the reaction rate could be made reasonably constant, greater precision could be obtained and smaller quantities of enzyme could be measured simply by increasing the duration of the reaction.

We had observed that the time-course of the reaction was the same when the serum and enzyme-labeled drug were added to the antibody simultaneously, as they were when the serum was added first, indicating that free drug reacts with antibody appreciably faster than enzyme-labeled drug. If we assume that the dissociation of the antigen-antibody complex is slower than the association reaction, it seemed possible to stop the reaction of the enzyme-labeled drug and the antibody in mid-course by saturating the remaining antibody sites with free drug.

We describe here our experience with a method of homogeneous enzyme immunoassay in which drug, antibody, and enzyme-labeled drug are allowed to react briefly, a large quantity of the drug then being added. The immunoassay, now based on that portion of the antibody sites that is most sensitively affected by the free drug in serum, has greater sensitivity. The enzyme activity, which is now relatively constant, is measurable with improved precision with any of several analytical instruments.

Materials and Methods

Reagents

The various reagents supplied in EMIT kits were reconstituted as recommended in the package insert. Vial A contains antibody to the drug of interest and the substrate to the enzyme label. Vial B contains enzyme-labeled drug, e.g., glucose-6-phosphate dehy-
Hydrogenase (EC 1.1.1.49) covalently coupled to phenobarbital.

Apparatus

A miniature Centrifugal Fast Analyzer (Oak Ridge National Laboratory) interfaced with a PDP/8e computer (Digital Equipment Corp., Maynard, Mass. 01754) was used as recommended (3) for enzyme assays. Unlike other centrifugal analyzers, this device employs a one-piece rotor that contains sample and reagent compartments and cuvettes, which is removed for loading and placed on the centrifuge-spectrophotometer for mixing and reading. The rotors we used had 17 cuvettes with 0.5-cm light paths that could accommodate 125 to 150 μl total reaction volume (4).

The rotors were loaded by use of the loading station (5), which includes two modified Automatic Pipettes (Micromedic Systems, Inc., Horsham, Pa. 19044) for dispensing and diluting specimens and reagents.

Rotors were loaded for EMIT assays by a minor modification of the procedure we used previously. Initially, 40 μl of standard, control, or patient’s serum was diluted with 200 μl of diluted buffer, mixed, and poured into the sample cup of the rotor loader. This device then dispensed 10 μl of the solution into the outer well of the rotor, followed by 22 μl of water. Simultaneously, 8 μl of concentrated buffer was dispensed into the inner rotor well and diluted with 23 μl of water. The rotor was then sent through the loader a second time to dispense 10 μl of the contents of vial A and 22 μl of water into the outer well and 10 μl of the contents of vial B and 22 μl of water into the inner well. During this procedure the drug contained in serum presumably had an opportunity to react with antibody, although mixing was probably incomplete. The total reaction mixture was 129 μl. Cuvette no. 1 contained water as a reference.

Reagents to be introduced after the first set of reagents were mixed were added to the cuvettes by “dynamic loading” (6), a technique that allows reagents to be added to all cuvettes while a reaction is in progress. A thin stream of solution was injected into the center of a spinning rotor through the 26-gauge needle attached to an SMI Micro-Petter (Scientific Manufacturing Industries, Emeryville, Calif. 94608). In this way, 250 μl of additional solution was distributed among the 17 cuvettes.

To determine the uniformity of distribution of solution to each cuvette, we used dye dilution. Rhodamine, sufficient to produce an absorbance of 0.3 at 550 nm, was added to the solution to be dynamically loaded. Rhodamine in this concentration produces negligible change in the absorbance at 340 nm and had no apparent effect on the antigen–antibody reaction.

At the end of the immunoassay, the transmittance of each cuvette was measured at 550 nm. We used the results from those cuvettes with transmittance that varied less than 15% from the mean. The final reaction volumes were therefore 144 μl.

A Model 24/25 spectrophotometer, with recorder, interfaced with a DP 3000 microprocessor (all from Beckman Instruments, Inc., Fullerton, Calif. 92634) was also used. In these experiments, 30 μl from vial A and 200 μl of buffer were added to 30 μl of standard (previously diluted by adding 30 μl serum standard with 200 μl of buffer) and 200 μl of buffer. Reagent B, 30 μl, followed by 200 μl of buffer was then added to start the reaction. Additional reagent was added with a SMI Micro-Re/Petter (Scientific Manufacturing Industries). The final 990-μl solution was then aspirated into the flow cell with 1-cm light path.

The DP 3000, which waits 6 s after the sample is aspirated into the cuvette, was programmed to wait an additional 10 s to allow for temperature stabilization, and then to record the activity of the solution seven times at 30-s intervals.

All experiments were run at 30 °C. Reagents were incubated at that temperature in a water-bath controlled to 30 ± 1 °C. The miniature Centrifugal Fast Analyzer controls the temperature to within ±1 °C, using a thermistor mounted in a cell in the rotor to provide a signal that controls a heating lamp placed above the rotor. The Beckman flow-through cell is temperature controlled to within ±0.1 °C. The DP 3000 microprocessor monitors the flow cell temperature independently and flags any deviation.

Absorbance changes ranged from 0.05 pmn for the zero standard to 0.18 pmn for the high standard where the time course of the reaction was followed.

Methods

The loaded rotor was placed on the analyzer and a plastic plate was positioned over the loading holes to prevent evaporation. It was then brought to 30 °C by slow rotation under the heat lamp. After the set-point temperature was reached, an additional minute was allowed to elapse for thermal equilibrium. The automatic logic circuit was then triggered. The rotor was accelerated to 4000 rpm, to transfer all solutions into their respective cuvettes, and then was stopped suddenly to mix all components. The rotor was then quickly brought up to "run" speed, 3000 rpm, and the computer began taking data as prescribed in the selected program.

The additional drug was introduced into the cuvettes by injecting a concentrated phenobarbital solution into the rotor center 10 s after run speed was attained.

To determine the time required for complete mixing, we loaded water into the reference cuvette and dye (p-nitrophenol) into the remaining 16 cuvettes. Water was then dynamically loaded. The reference cuvette absorbance was thus unchanged. The absorbance at 405 nm, recorded at 3-s intervals, became constant after 45 s. Mixing was considered to be complete at that time. A manual braking step which would have decreased mixing time was not included. The run-to-run time variation present in such a procedure introduced unacceptable reproducibility.

Because of excessive electronic noise at 3000 rpm, the speed was reduced to 600 rpm 55 s after the beginning of dynamic loading.
Results

Addition of excess phenobarbital to an EMIT assay for serum phenobarbital that is already in progress produced effects that depended somewhat on the serum phenobarbital concentration, i.e., the concentration originally mixed with antibody. When no phenobarbital was present, addition of excess produced a significant increase in activity for the first minute. The activity then stabilized at the higher value. Addition of an equal volume of buffer under the same conditions did not change the activity, which had stabilized (Figure 1, both charts, lowest curves). It should be noted that the activity during the first 100 s after reagents were mixed was not recorded in this experiment.

In the assay of serum standard containing 80 mg/liter, addition of excess phenobarbital increased the activity at the 100 s point from 55 to 70 U/liter. Over the next 4 min the activity decreased by about 6%. Addition of buffer permitted the usual 25% decrease in activity over the next 4 min (Figure 1, both charts, upper curves.)

When the 10 mg/liter standard was assayed, the effect of phenobarbital addition was intermediate. Compared to the buffer injection, phenobarbital increased the initial activity and stabilized it (Figure 1, both charts, middle curves). The effect of additional phenobarbital was to move the activity of this assay of an intermediate concentration further away from the zero concentration activity and closer to the higher concentration. The "standard curve" was thus changed to one yielding higher sensitivity.

The effect of adding phenobarbital was a function of the quantity added (Figure 2). Keeping the reagent concentrations as recommended by Syva, about 8 mg of additional phenobarbital per liter produced a maximal effect. To ensure this effect, we added enough to produce a final concentration of 22 mg/liter.

The timing for adding excess phenobarbital after the start of the assay was also significant. As the time was increased from 15 to 30 to 60 s, the activity at each serum standard concentration decreased. The curve for activity vs. time for the intermediate concentration became closer to the zero control, thus indicating decreased sensitivity.

The activity increased during the first 2 min, particularly when lower concentrations were being assayed. This effect was more pronounced as the time of addition of excess phenobarbital was increased (Figure 3).

A similar study of the effect of excess theophylline on the EMIT theophylline assay produced similar results (Figure 4). Excess theophylline was added to the reaction mixture 30 s after the addition of the theophylline-coupled glucose-6-phosphate dehydrogenase, instead of the 15 s used in the phenobarbital assay. The theophylline concentration, in moles per liter, needed to saturate antibody sites was equal to that of phenobarbital used in the phenobarbital assay.

Stabilization of the enzyme activity permitted kinetic
Fig. 3. Activity of the phenobarbital-enzyme complex vs. time, varying the time of addition of antibody-saturating quantities of phenobarbital

assays with repeated measurements and therefore increased precision. It also permitted phenobarbital to be assayed in an end-point procedure. The reagents and sample were diluted to 1/7 that recommended by the manufacturer, excess phenobarbital was added 15 s after the start of the reaction, and the absorbance, measured after 25 min reproduced the characteristic curve for activity vs. concentration (Figure 5). This permits conservation of expensive reagents.

Discussion

In sequential addition immunoassay, the interval between addition of labeled antigen and the time the label is to be assayed must be closely controlled. If the association reaction is not stopped, as in the homogenous enzyme immunoassay, the amount of time available to make the measurement may thus be limited. In the EMIT assay for phenobarbital, for example, the longer the time required for measuring the enzymatic rate, the lower the sensitivity of the immunoassay.

In the work described here we effectively stabilized the rate of the enzyme-catalyzed reaction by introducing a large quantity of free antigen and thus saturating the antibody. After the rate was stabilized, it could be measured over a longer period with the consequent predictable increase in precision and reliability of result. Smaller concentrations of enzyme could be measured, allowing use of less reagent (and sample) in performing the same assay. Smaller differences in enzyme activity could be distinguished, permitting higher precision of estimation of antigen. End-point enzyme assays, requiring, as usual, greater quantities of product formed for equivalent precision, could be used. Automating the assay using equipment customarily used for end-point assays is thus more feasible. Stopping the reaction of enzyme-coupled antigen with antibody in mid-course by adding excess free antigen was possible, because the rate of association of free antigen with antibody is faster than that of enzyme-coupled antigen, and enzyme-coupled antigen dissociated from the antibody to only a small extent in the time periods used.

In the EMIT assay there is sufficient antibody present to bind all the enzyme-labeled drug and many times the quantity of free antigen that usually is present (2). The marked effect of a small amount of free antigen on the rate of association of enzyme-coupled drug strongly suggests that the affinities of different antibody sites are very different and that the first sites to react with antigen are few in number and react rapidly. If the re-
action is stopped early in its course, small changes in unlabeled antigen present on the antibody produce larger changes in enzyme activity. Use of only these rapidly reacting sites thus gives rise to increased sensitivity, here defined as the ability to distinguish smaller changes in concentration at low concentrations.

The time courses of the reactions after addition of saturating antigen when different quantities of antigen were originally present were different. All showed an increase in activity, consistent with the idea that some of the enzyme-labeled antigen bound to antibody was released. More seemed to be released when there was little unlabeled antigen originally present. When concentrations of unlabeled antigen were high, the activity increased slightly, then decreased slowly with time. All of these changes seemed to be consistent with many different reactions occurring simultaneously at antibody sites with different affinities for labeled and unlabeled antigen. However, all the changes in activity were small as compared to those in the usual EMIT procedure.

We believe the technique described thus offers two main advantages. It permits that small fraction of all of the antibody sites that offer highest sensitivity to be selectively used. This improves the sensitivity of the sequential addition immunoassay technique. The approach should be applicable to other forms of sequential addition immunoassay where increased sensitivity is desirable. Secondly, in the homogeneous enzyme immunoassay, by stabilizing the enzyme activity, it permits the enzyme activity to be measured for a longer period, thus permitting us to take greater advantage of the enzyme as a label.

References