Computer-Controlled Automation of Radioimmunoassay Based on Gel Entrapment of Antibody

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We describe a new, broadly applicable approach to radioimmunoassay. Solid-phase binding reagent is prepared by entrapping antibody in polyacrylamide gel. This gel is then fragmented, sieved, and dehydrated in 95% ethanol. Upon evaporation of the ethanol, the dry antibody-gel particles are dispensed into miniature disposable plastic columns. A unidirectional flowing stream configuration is used to construct a standard curve for the polypeptide hormone, angiotensin I, used here to exemplify the technique. Good reproducibility of this standard curve is demonstrated. The analytical system includes a computer-controlled sample turntable, digital pipet, and liquid-switching assembly.

Additional Keyphrases: angiotensin I • radioimmunoassay of low-molecular-weight haptens

Radioimmunoassay (RIA) in the research and clinical laboratory has been characterized by arduous manual pipetting and by prolonged incubation and centrifugation steps. In this report we describe a rapid, broadly applicable, automated system of radioimmunoassay. This automated system is based on a unidirectional flowing-stream configuration of analysis, with use of miniature columns filled with a solid-phase gel-antibody reagent, which we have previously described (1, 2). The automation is a modification of the WISDOCS system described earlier (3, 4).

Materials
Antibody–Gel Binding Reagent

The technique for preparation of the gel-entrapped antibody reagent has been previously reported (1, 2). Briefly, the antiserum is added to a polyacrylamide gel preparation and photocatalytic polymerization is induced. The concentration of gel monomer and cross-linking reagent determines the pore size of the gel matrix. By selecting a gel recipe that results in a 250 g/liter content of polymer, the resulting pore size is such as to entrap the antibody molecule, yet allows the hormone to completely permeate it. High-molecular-weight interfering substances such as proteolytic enzymes and endogenous antibody are excluded from the intra-gel site of antibody binding (2).

After the antibody is entrapped, the gel is mechanically fragmented and wet-sieved to a 40- to 60-mesh particle size. After washing for 1 h in phosphate buffer (0.01 mol/liter, pH 7.4), the gel particles are dehydrated in 95% ethanol and air dried. The dehydration step collapses the gel particles to about a third of their wet volume. When rehydrated, the gel particles rapidly expand with no loss of binding activity. The shelf life of the antibody gel reagent in the dry state appears to be indefinite (2).

Standard and Labeled Hormone

Standard angiotensin I was purchased from Schwarz/Mann, Orangeburg, N. J. 10962, and I125-labeled angiotensin I (617 Ci/g) from New England Nuclear, Boston, Mass. 02118. All dilutions of standard and labeled hormone were made in the phosphate buffer to which had been added 2.5 g of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo. 63178) per liter.

Methods

A manual method of RIA in which the antibody gel reagent is used has been previously reported (2). Small, molded plastic columns have since been developed and are now available commercially (Engineering Industries, 6009 Nesbitt Road, Madison, Wis.). Each column (Figure 1) consists of a body and cap, which snap-fit together, with the Luer-Lok press fits shown. Screens, injection-molded into the top and bottom of the column, retain all particles with a diameter of 120 μm or larger.

The columns are prepared by first soaking them in a detergent solution, rinsing in distilled water, and drying with acetone. A 63-mg aliquot of the dry antibody-gel particles is accurately dispensed into the lower section of each column according to the “Accofil” dispensing principle (2), with a precision of ±0.7 mg (mean, 62.9 mg; coefficient of variation 1.1%; n =

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Received Feb. 5, 1975; accepted Mar. 12, 1975.

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33). The dispenser is commercially available (Perry Industries, Inc., Hicksville, N. Y.).

Once the dry gel is dispensed, the top of the column is snapped into place. When swollen by rehydration, the 63 mg of dry gel particles will just fill the internal cavity of the column. This rehydration requires a total volume of 0.30 ml of solution, of which 0.18 ml is required for the rehydration and 0.12 ml fills the void volume between the gel particles.

Automation of the gel RIA technique requires a precisely timed system that is capable of dispensing and collecting many solutions. Such a system, developed for batch automation of chemical assays, has been previously described (3) and evaluated (4). This system, as utilized here, is shown in Figure 2. It includes a PDP-12/30 computer (Digital Equipment Corp., Maynard, Mass. 01754) and appropriate interfacing to control a digital pipet (5-ml capacity, 5-μl increments); a 24-port fluid switching valve (Scannivalve, Inc., San Diego, Calif.); several three-port, two-position, solenoid-driven valves (Skinner Valves; Norman Engineering Co., Inc., Chicago, Ill.); and a sampler turntable.

The software required to operate this system under computer control has also been described (3, 5). The programming language, POLAC (Problem Oriented Language for Analytical Chemistry) was originally designed and written for discrete spectrophotometric analyses. Both the hardware and software were easily adapted to the problem of automating RIA.

Figure 2 illustrates the overall experimental sequence for an automated RIA run. The columns are filled with an accurately dispensed amount of dry gel particles, then are connected to alternate ports of the 24-port fluid switch. Silastic tubing (E. I. du Pont de Nemours and Co., Inc., Wilmington, Del. 19898) proved superior to Teflon and polyethylene tubing with respect to minimizing nonspecific binding of radioactive hormone and so is used for all connecting lines. A 5-ml disposable polypropylene syringe (“Stylex” syringes; Pharmaseal Laboratories, Glendale, Calif.) is press-fitted to the top of each column and serves as a fluid-collection reservoir. A drain line of Silastic tubing is inserted into each syringe reservoir and connected to the valve port adjacent to the column input. A second line, used for automated cleaning of the syringe reservoir, is also inserted and connected to a three-port, two-position valve. The automated syringe-cleaning technique is described below.

All solutions are added unidirectionally and sequentially to the columns. The effluents are collected in the 5-ml syringe reservoirs as they appear at the top of the columns. The angiotensin I samples and standards are placed sequentially in the sample turntable, followed by an equal number of counting vials for the eventual collection of both the unbound and bound fractions of radioactively labeled hormone. Stock solutions of I\(^{125}\)-labeled angiotensin I, HCl (10 mmol/liter, pH 2), and albumin/phosphate buffer are also connected to the 24-port fluid switch by Silastic tubing. The program is then started and no further manual intervention is required except for the final step of placing the counting vials in the gamma counter.

Computer Program

The logic sequence is illustrated in Figure 3. The program asks, by way of the Teletype, for four initial pieces of information: the number of standards, the number of samples, the headstart sample incubation time (headstart time is the interval between addition of sample and addition of radiolabeled hormone), and the incubation time for the competition between sample (or standard) and the I\(^{125}\)-labeled hormone. With this information imparted, the program calls for calculation of all required variables and priming of all connecting lines to the reagents.

First, the pipet-holding coil and the sampler line are thoroughly flushed automatically with distilled water. The standards and (or) samples are then sequentially collected from the turntable and added, in excess, to the appropriate column. The volume of sample added is determined primarily by the water regain of the dry gel particles. Thus the accuracy of sample addition depends on the accuracy with which the dry gel particles are dispensed rather than on the accuracy of the digital pipet.

When fully hydrated, the gel particles just fill the
column. Enough sample is delivered to overfill the column slightly; the excess is expelled through the upper screen into the collection reservoir. This excess sample cannot compete for the gel-entrapped antibody binding sites. The time required for sample addition is accurately controlled by the computer. The holding coil and sampler line are automatically and thoroughly cleaned between each sample addition.

When sample has been added to all columns, the holding coil is filled with the competing radiolabeled hormone. When this has been completed the total elapsed time is compared to the requested headstart incubation time. If the headstart time has not been exceeded, then the program causes a wait for the remaining time. Otherwise the sequence will immediately continue to the next process.

The labeled hormone is sequentially added to each column, also in excess. This step requires only 2 s. This operation effectively flushes the void volume of the column. Competition for the gel-entrapped binding sites begins immediately. The headstart time is identical for each column.

When all columns have received the 125I-labeled hormone, the small volumes of sample (or standard) hormone plus labeled hormone that have accumulated in the collection reservoirs are simultaneously and automatically emptied and rinsed as follows. Under computer control, a three-port, two-position valve is opened to allow buffer solution to fill the syringe reservoirs, by use of the cleaning lines described earlier. A solution-level detector is placed near the top of one of the syringe-reservoirs. This detector automatically closes the rinse valve when the syringe-reservoirs are full, and simultaneously opens the cleaning lines to vacuum pump suction for a timed interval (15 s) to drain the reservoirs completely. This cycle is repeated three times, to clean and finally to empty the reservoirs.

When the cleaning process has been completed, the holding coil is filled with albumin/phosphate buffer. The program then checks the elapsed incubation time with the value entered by the user. If necessary, the remaining time is allowed to pass before going on to the column-wash cycle. The total incubation time for all RIA determinations reported here was 20 min. This remarkably short incubation time is feasible because: (a) Most RIA methods begin with a dilution of the sample or standard. This dilution step is either inherent in the RIA method or necessary to "dilute out" inhibitors. In our method, dilution is eliminated because the dehydrated gel-antibody is rehydrated in the sample volume, and high-molecular-weight interferences are excluded from the intragel compartment. (b) Short incubations are valid if incubation time is precisely controlled. With this automated RIA system, the competitive reaction system need not go to completion because each column is treated identically.

Free and bound hormone are separated by flowing buffer solution through the columns. This is, again, done sequentially. The first column receives three consecutive injections of 250 µl, each 250-µl bolus being separated by 30 s to allow for diffusion-limited washout of unbound hormone from the intragel compartment. This entire sequence is repeated three times. Each column is therefore washed with a total of 2.25 ml of buffer solution, which is collected in the 5-ml syringe-reservoir.

When the wash cycle has been completed, the effluents containing the unbound fraction are sequentially collected for processing. The effluent is aspirated into the holding coil, the turntable is advanced, and the wash solution is delivered into a clean 3.5-ml counting vial. An additional 1.0 ml of buffer solution is also delivered, to effectively clean the holding coil and sampler lines, thus assuring complete solution delivery. These vials are then transferred manually to a "Biogamma" gamma counter (Beckman Industries, Inc., Lincolnwood, Ill.).

In our initial experiments the columns were themselves counted at this point, to determine the bound radioactivity directly. Further experimentation has shown that the bound activity can be eluted from the column by repeating the buffer wash and collection steps, with use of 10 mmol/liter HCl (pH 2) instead of the buffer (2). This provides a totally automated collection of the bound activity, in solution form. No manual manipulation of the column is necessary.

Fig. 3. Flow chart for automated radioimmunoassay sequencing. See text for details.
Results

Figure 4 shows typical working curves obtained with the automated RIA process described above. Each plot shows the symmetrical curves obtained for the bound (upper curve) and unbound (lower curve) fraction for the clinically useful range of angiotensin I when assaying for renin activity. Figures 4a and 4b also show the values for total activity, obtained by adding the bound and free values.

The upper curve in Figure 4a was determined by counting the columns immediately after separation and collection of the unbound fraction. The upper curve in Figure 4b was determined by collecting the bound fraction as an acid wash effluent. Figure 4c presents the same data as 4a, but shows the curves as a fraction of the total labeled hormone added to the column. The total activity (100%) was determined by averaging the seven values shown in Figure 4b (mean, 19400; SEM, 82 counts/min).

Figure 4d shows the results of three separate runs performed 12 h apart with use of the same solutions of standard and labeled hormone, but with duplicated columns. Again, the bound fractions were determined by use of an acid wash. The error bars around each point indicate the standard error of the mean for the three values obtained.

Discussion

The system described here is the product of our initial effort to automate radioimmunoassay definitively. We used only instrumentation immediately available to us—a liquid-switching assembly, a preci-
tion digital pipet, and a sample turntable—all under computer control. This system was originally developed for automated discrete spectrophotometric analyses (3, 4).

In this approach to automated RIA, only one pipet is used; it must, for each determination, sequentially pick up, deliver, and carry away a variety of solutions: sample or standard, labeled hormone, buffer, acid, and column effluents. Cross-contamination between these solutions has been a major problem. However, we find that this type of chemical carryover is eliminated by the dual strategy of multiple wash steps and air bracketing in the holding coil to prevent mixing from laminar flow. Standards differing in concentration by 10,000-fold can be analyzed consecutively without evidence of significant carryover.

At present, the digital pipet limits sample throughput because of its large duty cycle. However, the same mechanical system and controller that operates one digital pipet can be easily modified to operate several pipets simultaneously.

The precision digital pipet we used was from a prototype Automatic Clinical Analyzer (E. I. du Pont de Nemours). It is not yet established, however, that such a high-performance pipet is necessary for automation of this type. As stated, volume of sample added is determined by the water regain of the gel, rather than by the accuracy of the sample addition. We have shown that over- or under-filling the column by as much as 10–20% has little effect, because slight underfilling does not change the amount of sample delivered to the intragel compartment and because the excess is merely forced above the upper screen of the column, where it cannot compete for the intragel binding sites.

Furthermore, diffusion is the limiting factor in the washing steps; therefore washing efficacy depends more on the time between wash additions than on the volume of the wash solution.

For these various reasons we conclude that a precision pipet is probably not necessary for this approach to automated RIA.

The sample-application step is not diffusion limited; it is rapid; the gel particles are rehydrated in about 10 s.

Radioimmunoassay frequently suffers from the presence of interfering substances of high molecular weight. In most RIA methodology these interferences are eliminated—for at least minimized—by diluting the sample. This, of course, slows the reaction and thus the incubation is prolonged. Furthermore, most RIA methods require that the incubation step be given enough time for complete equilibrium to be established.

With the automated techniques described in this report the incubation steps can be terminated after a precise interval, well before equilibrium is reached. Thus, a more rapid response time can be achieved. The effect of 5-, 15-, 30-, and 60-min incubations at room temperature on the angiotensin I assay has been previously studied (2). Binding was 57.0, 74.3, 83.3, and 92.0% respectively, of that obtained at 18 h. This favorable kinetic picture validates short (for data presented in Figure 4, 20-min) incubation times as long as these times are accurately controlled and each column is treated identically.

The column design shown in Figure 1 was chosen after observing the flow patterns of colored solutions through columns of differing design. This design offers the most even perfusion and minimizes bubble formation in the column.

This approach to RIA automation will become more attractive when solid-phase antibody binding reagent is made reusable. Reusability based on acid elution of bound hormone followed by dehydration of the gel–antibody particles in acetone and then drying by pumping air through the column appears promising; we currently are evaluating this approach to reusing the columns.

This work was supported by NIH Research Grants GM-10978 and GM-16133. S. J. U. is a recipient of an NIH Research Career Development Award. We thank Dr. Ted Goodfriend for his generous gift of antiangiotensin antisera.

References