Gel Entrapment of Antibody: A New Strategy for Facilitating Both Manual and Automated Radioimmunoassay

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A technique of radioimmunoassay is presented that eliminates pipetting and centrifugation, and excludes interferences by high-molecular-weight materials from the incubation and separation steps. A solid-phase binding reagent is prepared by first entrapping antibody in polyacrylamide gel. This gel is then fragmented, sieved, dried with ethanol or lyophilized, and placed in miniature disposable chromatographic columns. Application of the sample to the intra-gel column compartment is determined by the water regain of the gel. This pipetteless method of sample application depends on reproducible aliquots of dry gel particles in every column. A method for preloading radiolabeled hormone and standard hormone into the column is also described. This technique has been successfully applied to the assay of angiotensin I and insulin. Dry antibody–gel stored at room temperature for 26 months has not shown loss of binding activity.

Additional Keyphrases: angiotensin I • insulin • RIA of low-molecular-weight hapten

Over the past decade, there have been a multitude of reported methods for radioimmunoassay (RIA) of polypeptide hormones of clinical interest. Of these, many are either too arduous or unreliable for widespread application in the clinical laboratory.

Here, we report a broadly applicable system, which substantially facilitates both manual and automated methods of RIA. This RIA system features (a) exclusion of interferences from high-molecular-weight materials, (b) elimination of any pipetting or centrifugation, and (c) long-term stability of the binding reagent stored at room temperature.

Materials

Antibody–Gel Binding Reagent

Antibody was immobilized in polyacrylamide as reported previously (1). Polyacrylamide, 20 g, and 5 g of N,N’-methylenebisacrylamide (Eastman Kodak Co., Rochester, N. Y. 14650) were dissolved in about 70 ml of phosphate buffer (0.1 mol/liter, pH 7.4). Antisera was added to this solution to the desired concentration, determined empirically, as described below. If a suitable concentration has been previously determined in another RIA system, then this concentration can serve as a guide for development of a gel-based RIA procedure. In our experience, we required a concentration of antisera in the gel mixture about 10 times that required for solution-phase assays with the same serum.

Riboflavin (Sigma Chemical Co., St. Louis, Mo. 63178) was added to give a final concentration of 1 mg/100 ml of gel, and the gel solution brought to a final volume of 100 ml with the phosphate buffer. Polymerization was initiated by photocatalysis with a No. 2 photo flood lamp, and was complete in 10 min. The gelation reaction is exothermic. A thermometer was placed in the gel. The temperature was kept at less than 60 °C by use of an ice bath during the polymerization. (Most antibody serological reagents have already been incubated at 60 °C for half an hour to inactivate complement.)

The antibody gel was fragmented by forcing it through a No. 40 mesh brass screen. Gel particles were collected on a No. 60 mesh screen and washed with about 5 liters of distilled and de-ionized water. The gel particles sedimneted rapidly and were stiff enough to tolerate moderate flow rates (1 to 4 ml/min) in a chromatographic column without plugging. After sieving, the gel particles were equilibrated with the phosphate buffer.

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Received July 23, 1973; accepted Sept. 17, 1973.
Gel Drying Procedure

Two fundamentally different drying procedures have been studied: (a) lyophilization, and (b) dehydration in 95% ethanol followed by air drying. Neither method was associated with loss of antibody activity. However, ethanolic dehydration was faster and simpler. The gel particles were suspended in five volumes of 95% ethanol and allowed to settle. The supernatant solution was discarded and the ethanol treatment repeated once more. The ethanol-dehydrated particles were then allowed to dry in room air by spreading them out on a nylon net.

Antisera have long been known to lose activity, even at 6 °C, if precipitated from aqueous solution by adding ethanol to a concentration greater than 25% (2). To our knowledge no one has previously reported that gel-entrapped antibody dehydrated with 95% ethanol regains all its binding activity upon rehydration. We attribute the lack of loss of antibody activity to gel entrapment, which prevents denaturation by preventing massive aggregation of the hydrophobic portions of antibody protein.

Upon rehydration these ethanol-dried particles behaved in an important and fundamentally different manner than did the freeze-dried gel particles. Microscopic observation revealed that the ethanol-dried particles were initially collapsed and then underwent rapid expansion during rehydration. On the other hand, freeze-dried particles were not collapsed and on rehydration did not expand, but instead released a shower of tiny air bubbles. Rehydration of both lyophilized and ethanol-dried particles was at least 90% complete at the end of 5 s.

Storage and Stability

Standard curves constructed from gel binding reagent show no change with respect to duration or temperature of storage. Lyophilized gel particles with entrapped anti-insulin antibody activity have been hermetically sealed and stored at 37, 25, 4, and −20 °C for over two years without detectable loss of antibody activity. Our six-month experience with ethanol-dried gel particles containing anti-angiotensin antibody has also shown stability to be satisfactory.

Antisera

Rabbit antisera against angiotensin I were induced as previously described (3). Guinea pig antiserum against insulin was the gift of Dr. Peter Wright, University of Indiana.

Standard and Labeled Hormone

Standard angiotensin I was purchased from Schwarz/Mann, Orangeburg, N. Y. 10962, and labeled angiotensin I from New England Nuclear Corp., Boston, Mass. 02118. Human insulin standard and 125I-labeled bovine insulin were obtained from Burroughs Wellcome, Inc., Beckenham, England, and Amersham/Searle, Arlington Heights, Ill. 60005, respectively. All dilutions of standard and labeled hormone were made in 0.1 mol/liter pH 7.4 phosphate buffer containing 2.5 g of bovine serum albumin (Sigma) per liter.

To make it unnecessary for the user to add radioactive labeled or standard hormone to the test system, we devised a method to incorporate these components in the dry gel particles. Peptide hormones are ethanol soluble. Once the particles with gel-entrapped antibody activity have been ethanol dehydrated and dried in room air, then peptide hormone can be incorporated by dampening these dry particles with a 95% ethanol solution of the hormone, followed by air drying these particles on a nylon net or “Parafilm” surface. By this procedure, the peptide hormone was incorporated in or on the gel particle, but was not complexed at the antibody binding site. Specific binding occurred only upon rehydration of the gel particles. The small amount of nonspecific binding of peptide hormone that occurs to the nylon net was minimized further by adding an ethanol-soluble peptide such as polymyxin to the ethanol drying reagent.

Methods

The dry antibody-gel binding reagent was dispensed volumetrically as diagrammed in Figure 1. Equipment based on the same principle is commercially available (Perry Industries, Inc., Hicksville, N. Y.). Aliquots of dry antibody–gel weighing 33 mg could be dispensed with an accuracy of ±0.3 mg (SD, 1%; n = 30). Aliquots as large as 67 mg or as small as 10 mg were also dispensed with an accuracy somewhat better and somewhat worse, respectively.

One-milliliter disposable tuberculin syringes were modified for use as miniature chromatographic columns as diagrammed in Figure 1.

Typically, 33 mg of the dry gel binding reagent was placed in each syringe column. This amount of dry gel particles has a water regain into the intra-gel compartment of 0.10 ml. To carry out a determination, enough sample or standard hormone was drawn into the syringe-column to rehydrate the gel (0.10 ml), fill the void volume between the gel particles (0.03 ml), and just cover the nylon net (0.13 ml total volume).

Fig. 1. Volumetric dispenser for antibody–gel binding reagent
(a) Constant suction device; (b) Apparatus for aliquoting dry gel particles; (c) Miniature syringe-column loaded with dry antibody-gel particles
Alternative methods were then followed: \((a)\) If the competing radioactively tagged hormone was already incorporated into the gel particles, incubation begins immediately; \((b)\) if the competing radioactively tagged hormone was not previously incorporated in the gel particles, this hormone solution was drawn quickly into the column in a volume in excess of one column void volume (i.e., \(>0.03\) ml). Note that the sample hormone can be given a "head start" as is commonly found useful in RIA. After an incubation of 15–240 min, at room temperature, unbound hormone was washed from the column with the albu-
mim-containing phosphate buffer solution described above.

Various washing procedures have been used successfully. Washout of the intra-gel compartment is primarily a diffusion-limited process, and therefore substantially independent of the amount of wash solution. Approximately 5 min were required for 90% of the hormone to be washed out.

The unbound hormone in the wash solution or the bound hormone on the column, or both, were count-
ed in an automated\(^1\) gamma counter. Placing the miniaturized column-syringe in the counting test tube and counting bound hormone proved to be the simplest manual procedure.

All samples and standards were run in triplicate and averaged. Labeled angiotensin and insulin were used at specific activities of 1000 and 50 Ci/g, respectively. About 10,000 counts were initially introduced into each test system. All assays were run at room temperature.

Results

Figure 2A shows typical standard curves obtained and the effect of changing the concentration of antibody in the gel recipe. The useful clinical range appears stippled. Standard curves for concentrations of hormone of several orders of magnitude difference are shown in Figure 2A. A narrower range of standard hormone concentration (and the standard error of the mean) is shown in Figure 2B. Figure 2C shows standard curves obtained exactly as those in Figure 2A, except that the pre-incubation of the standard hormone before addition of labeled hormone was 30 s instead of 30 min. Figure 2D shows a standard curve generated with labeled hormone incorporated in the dry gel particles before the gel particles were dispensed into each column. Enough gel reagent was prepared to run two standard curves 20 weeks apart.

Note in Figure 2D that these curves are identical, despite the fact that the columns used for repeat assay had been stored at room temperature for 20 weeks. (Because of the decay of radioactive label, the columns used after 20 weeks of storage were each counted for 5 min instead of 1 min.)

The effect of 5-, 15-, 30-, and 60-min incubation time on angiotensin assay was studied; there was, respectively, 57.0, 74.3, 83.8, and 92.0% of the binding obtained at 18 h, with use of labeled hormone alone.

Discussion

Sample application is controlled by the water regain of reproducible dry aliquots of antibody-gel particles. Over-filling the column-syringe by as much as 20% of the column volume does not alter results. In other words, hormone sample pulled above the nylon net does not compete for intra-gel antibody binding sites, which are all located below the nylon net. This assumes that the nylon net (pore size: 37 \(\mu\)m) acts as a reasonably good anticonvection barrier and that the column-syringe is not agitated during incubation. Similarly, under-filling the column does not alter the results, provided the deficit is in the extra-gel volume and all of the gel is hydrated. This degree of permissible under-filling also is about 20% of the dry gel volume.

Pipetting of the sample is not necessary by this
technique, *not* because a syringe can be used as accurately as a pipet, but rather because dry gel particles can be aliquoted very accurately and sample application is precisely determined by the water regain of the intra-gel compartment.

**High-Molecular-Weight Interferences**

Radioimmunoassay methods are subject to a variety of interferences. Generally, standards run in plasmas that are rendered hormone free and that are obtained from different subjects vary substantially (4). This variation is attributed to unknown interferences. For this reason, for example, measurements of angiotensin II in plasma have to be controlled by simultaneous standard curves diluted in the same plasma, which has been rendered angiotensin free (5). Removal of all interferences, which is necessary for absolute measurements of hormone, is arduous. Dilution has been a standard technique for reducing the effect of these interferences. However, when the assay must be carried out at the limits of the sensitivity of the method (picograms per milliliter of plasma), then dilution can no longer be used (5). Many of these interferences are poorly characterized and referred to simply as inhibitors, enhancers, proteases, or factors such as “insulin resistance factor.”

Radioimmunoassay for insulin in plasma from insulin-treated diabetics is complicated by the presence of endogenously produced antibody against porcine or bovine insulin, various techniques to eliminate these interferences have been reported, but none has received widespread acceptance (6–10). Similarly, radioimmunoassay of angiotensin is complicated by the presence of converting enzyme, angiotensinases, high-molecular-weight binding molecules, and other less well-defined interferences (5, 11–13).

Many of the substances causing these interferences are of high molecular weight. Therefore, we propose a combined incubation and separation step based on gel permeation that excludes high-molecular-weight interferences in the sample. By trapping the antibody inside a gel that allows access according to molecular weight, an inner reaction phase is separated from an outer ambient phase. Molecules that are small relative to the pore size will have free access to the inner and outer phases. In theory, high-molecular-weight interferences that interacted with the small molecules of labeled or unlabeled antigen would be able to interact with that portion of the small molecules in the ambient phase. In time, all molecules would reach both the outer and inner phases. Thus, at equilibrium, there would be no theoretical benefit from separating the interferences from the antibody by a barrier permeable to the antigens.

The system described in this report improves on the earlier system (1), in which a permeability barrier was the only influence retarding interference. By using dried gel with entrapped antibody, a near-instantaneous separation is achieved, admitting to the inner phase those low-molecular-weight molecules in the sample along with the water of hydration of the gel. Although there is still an outer phase, now containing any interferences in very high concentration, it is separated from the antigen by longer distances, and therefore by time of diffusion. In other words, the antigen can react with the entrapped antibody for a longer time and over a shorter distance than it has to react with the concentrated interference phase outside the gel. By interrupting the reaction before equilibrium is achieved, the accessibility of the antigen to antibody is made more significant in the overall reaction than it would be if longer times were used.

Furthermore, the outer extra-gel compartment, which typically has a volume of 0.03 ml, can be rapidly flushed with buffer solution. For example, two extra-gel void volumes (0.06 ml) can be pushed through the column in less than 2 s, which theoretically should remove any high-molecular-weight interferences that were sequestered and concentrated in the extra-gel void volume during hydration with sample. It is true that a quick flush of the outer extra-gel compartment will also remove a small portion of the intra-gel compartment—that is, that portion of the intra-gel compartment that can diffuse out into the extra-gel compartment during the 2-second flush procedure. How small a portion of the intra-gel compartment is lost during this quick flush procedure will depend primarily on the gel particle size and duration of the flush step. Because 90% washout of the intra-gel compartment takes 5 min in our system, we conclude that a 2-s flush step does not entail significant loss from the intra-gel compartment. Furthermore, this small loss should be the same for every column, particularly in an automated system based on computerized control of a precision digital syringe pump.

The molecular-weight exclusion-threshold for gel permeation is determined by the amount of gel polymer and cross-linking reagent used in the gel recipe. Control of gel pore size is well-understood theoretically (14, 15), and can be measured experimentally by various gel permeation and permeability techniques (16). Exclusion of high-molecular-weight interferences in use of gel RIA is only applicable when the interfering substances have a substantially greater molecular weight than does the hormone or hapten in the assay system. Thus, this concept can be applied to the RIA of polypeptide hormones, steroid and cyclic fatty acid hormones, drugs, and other haptenes, but cannot be applied to large antigens such as the Australia antigen.

Studies to validate the concept of excluding high molecular weight interferences during gel-antibody RIA have been made. Non-heparinized plasma samples were obtained from four hemodialysis patients. These patients were all surgically anephric and therefore lacked both the enzyme renin and the product of renin activity, angiotensin I. Standard curves for angiotensin I were run using both the gel
and standard charcoal assay as modified by Goodfellow (5, 17). In both these methods, converting enzyme and some of the angiotensinase activity is inhibited by the addition of ethylenediaminetetraacetate (2.6 mmol/liter), dimercaprol (B.A.L.; 1.6 mmol/liter), and 8-hydroxyquinoline (3.4 mmol/liter) (11). The remainder of the angiotensinase activity was inhibited by adding phenylmethylsulfonyl fluoride (0.5 mg/ml). All data points were the average of triplicate or quadruplicate determinations. The resulting standard curves for both RIA systems were analyzed statistically (F-test) (18) for significant differences created by various plasmas. At the .01 significance level, there was no difference in the four plasma curves assayed in the gel system, suggesting that individual plasma interferences were eliminated. The standard curves obtained by the charcoal method showed a significant difference at the .05 level, which we attribute to the different plasmas.

A severe test of the gel protection of both antibody and antigen from high molecular weight interferences was designed. Chymotrypsin degrades angiotensin (19). When alpha-chymotrypsin (Sigma Type I) was preincubated at a concentration of 20 μg/ml with angiotensin I for 1 h at 25 °C in the phosphate buffer solution containing albumin, then 63% of hormone binding activity was destroyed compared to the control incubation without chymotrypsin. When gel–antibody particles were preincubated in this concentration of chymotrypsin for 1 h and then washed and used for gel RIA, we saw no decrease in binding activity compared to appropriate controls. This concentration of chymotrypsin was also introduced into the gel RIA system at that moment when standard angiotensin, labeled angiotensin, and dry gel-engaged antibody contacted one another. After 1 h of incubation, a substantial (87%) protection was shown. That there was not 100% protection may be attributed to the fact that chymotrypsin is a relatively low-molecular-weight (mol wt, 23,000) enzyme, and thus may have penetrated into the gel to some degree, or the chymotrypsin may have destroyed hormone in the extra-gel void volume, which was not flushed in these studies. More extensive studies of the gel method of excluding high-molecular-weight interferences are planned.

Automation

Antibody gel RIA, as described in this report, presents advantages for both manual and definitively automated RIA systems. Because the system is based on a column-chromatographic unidirectional flowing stream configuration of analysis, automation requires basically only addition of a precision syringe or proportioning pump, a detection system, and a processor-controller. We envision automation based on a manifold of several dozen disposable or reusable miniature columns filled with antibody gel particles. An automated system would provide highly reproducible application of sample, reagents, and wash solution to the miniature columns. Both unbound and bound radioactivity can be determined automatically by elution from the column into a detector with a flow-through cuvette. Unbound radioactivity is washed off the column by a variety of physiological solutions at neutral pH, while bound radioactivity can be eluted off the column with acid or hyperthermic wash solutions as shown in Figure 3. These column eluents can be analyzed in either gamma detectors or liquid-scintillation detectors. The ability to exclude high-molecular-weight interferences and to work with a highly stable prepackaged antibody–gel reagent appears advantageous for both manual and automated approaches.

Manual methods of RIA have generally required incubations of many hours in order for the system to reach equilibrium. However, gel–antibody RIA does not require dilution of plasma samples to reduce or eliminate high-molecular-weight interferences. Thus, reaction kinetics proceed toward equilibrium faster. Furthermore, reaching equilibrium before carrying out the separation of bound from unbound hormone is not necessary. Nonequilibrium techniques usually allow interaction of the antibody with antigen in the sample before labeled hormone is added. The purpose of this manipulation is to increase the sensitivity of the assay.

Two features of radioimmunoassay make it customary to allow long times of incubation before separating bound and free labeled hormone. The first is the fact that antisera are heterogeneous; only a fraction of antibody molecules have very high relative affinity for antigen. The high-affinity antibody molecules are the ones that lend the highest degree of sensitivity and accuracy to the assay. Long times of incubation are required before the minority of high-affinity molecules can exert their full influence on the bound:free equilibrium.

The second feature that dictates relatively long times of incubation is the time of reagent addition. If the first tube in a set receives the final reagent long

![Figure 3. Elution of bound labeled angiotensin I as a function of pH and temperature](image-url)
before the last, but all are subjected to the separation step together, and all are separated before equilibrium is achieved, early and late tubes may be incubated for significantly different times. As incubation times decrease, this discrepancy becomes more and more important. However, an automated system such as described above would make possible relatively short but precisely timed incubation and wash steps.

References