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A BIASED SOLID-PHASE RADIOIMMUNOASSAY FOR THYROXINE (T_4) USING T_4-^{125}I PRESATURATED ANTIBODIES

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An assay for thyroxine (T_4) is demonstrated using specific antibodies covalently coupled to controlled pore glass (CPG). In this assay immobilized antibodies saturated with labeled T_4 are employed in a preloaded unit tube configuration. These complexes are stable for long periods of time when stored in buffer. This concept results in a highly reproducible and sensitive assay for T_4 that requires a single pipetting step.

INTRODUCTION

In recent years the use of immobilized antibodies in radiological assay systems has become more frequent (1,2). Such systems are attractive because they not only reduce the number of pipetting steps and reagents needed in the assay but also simplify the separation of bound from free labeled antigen. This procedure generally consists of a filtration or centrifugation. Although the total time for such assays has in many instances been drastically reduced, the need for separate pipetting steps for both sample and labeled material still exists. This report deals with an assay for thyroxine (T_4) using specific antibodies immobilized on porous glass particles that have been prepared with an optimal quantity of labeled antigen already bound to the antibodies. This type of "biased" assay has several advantages: a reaction tube containing optimal quantities of labeled antibodies can be prepared in advance and stored without deterioration; separate pipetting of labeled antigen is eliminated, thus reducing both error and the time necessary for the assay procedure; and the assay, in addition to being rapid, is extremely sensitive and can be used with a variety of sample sizes ranging from 10 to 100 μl.

MATERIALS AND METHODS

Preparation and Handling of Antisera

Antisera to T_4 were prepared in rabbits using a T_4 conjugate of bovine serum albumin that had been prepared with water-soluble carbodiimide (3). Antisera of suitable titer ($\approx 1:10{,}000$ or greater) were obtained in the animals approximately three months after initial injections of 1.0 mg of the conjugate in Freund's complete adjuvant. The sera were decomplemented for 0.5 h at 56°C and stored at −20°C.

Preparation of Immobilized Antibodies (IMA)

Controlled-pore glass of 1 μm particle size was derivatized to contain an aryl amine functional group (4). One gram of this glass was reacted with nitrous acid to yield a diazonium glass that was then reacted in the cold with 2.0 ml of the whole antiserum. The pH of the reaction was measured using β-naphthol. After coupling was complete, the glass derivative was exhaustively washed with 1% saline solution and PBS–BSA (0.03 M phosphate, 0.15 M sodium chloride, 0.1% bovine serum albumin, pH 7.5).

Preparation of Prelabeled Assay Tubes

An initial titer curve using the immobilized antiserum (IMA) was obtained by the serial dilution of 0.1 ml of the derivative in 12×75 mm polystyrene tubes. To 0.1 ml of the diluted IMA was added 0.8 ml of PBS–BSA, 2.5 mg of merthiolate, and 0.1 ml of labeled T_4. From the initial titer curve, a quantity of IMA (≈ 100 mg) was incubated with saturating quantities of labeled T_4 obtained from Corning Medical Co., Medfield, Massachusetts. The IMA was diluted to a concentration of 20 mg/ml. Enough labeled T_4 was added so that, at the proper concentration of IMA, a total of 20,000 cpm would be added per tube. The label was permitted to saturate the antibody by incubation in PBS–BSA, at pH 7.2 and 37°C for 3 h. The mixture was centrifuged at 5,000 rpm for 15 min and decanted, and the derivative was washed twice with PBS. The IMA was resuspended in PBS–BSA at pH 7.2 containing 1.3 mg merthiolate so that each tube received 0.8 ml of suspension and gave approximately 10,000 cpm. The tubes were stored at 4°C until used.

Preparation of Dose-Response Curves

In order to determine the appropriate serum sample size for the T_4 assay, a series of dose-response curves was constructed in the following

fashion: T_4 standards (0–200 ng T_4 ml) were prepared in PBS–BSA. To preloaded tubes containing the saturated IMA were added T_4 free serum (3) and T_4 standards in volumes of 0.01, 0.025, 0.05, 0.1, and 0.2 ml. The tubes were incubated at 37°C for 2 h with a vortex after 1 h. They were then centrifuged at 5000 rpm for 10 min and decanted, and the glass precipitate was counted for 1 min.

RESULTS

Figure 1 shows dose–response curves as a function of serum sample size. An equivalent quantity of T_4 free serum was included in each tube. It can be seen that the best sensitivity and range were found between a sample size of 10 and 50 μl. The inter- and intra-assay reproducibility for repeated assays run on the same day with the identical reagents averaged ±5%. The variability between runs on consecutive days increased to ±8%. However, the intra assay variability remained constant at ±3%. The slightly increased variation between assays on consecutive days may be related to the activity losses noted over time during the phase of storage (Table 1). We did not repeat these studies with prestored material other than what was necessary to show stability.

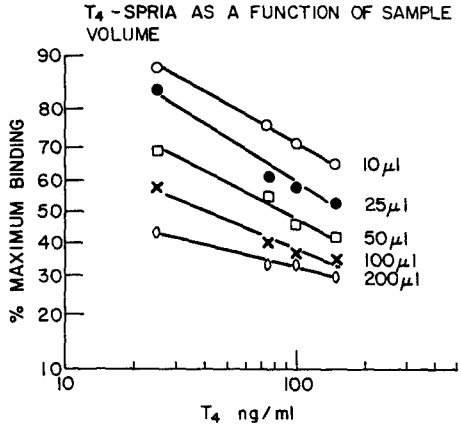

FIG. 1. Increasing quantities of T_4-free serum and T_4 were added to each tube for this dose response curve. Results showed a depression in maximum binding and a decreasing slope in the response curve.

TABLE 1. **50°C Storage Stability of Preloaded Unit Tube**[a]

Hours	Bound at 0 ng/ml T_4 (%)	Bound at 100 ng/ml T_4 (%)
0	62	26
24	56	24
48	55	32
72	55	32

[a] In the presence of 0.05 ml T_4 free serum.

DISCUSSION

The use of bias assay systems to achieve maximum sensitivity in classical radioimmunoassay procedures is well known. In general, the mass of labeled antigen in these systems is very small when compared with any of the concentrations of the specific substance to be measured. In these instances, one generally allows the labeled antigen to bind maximally followed by the

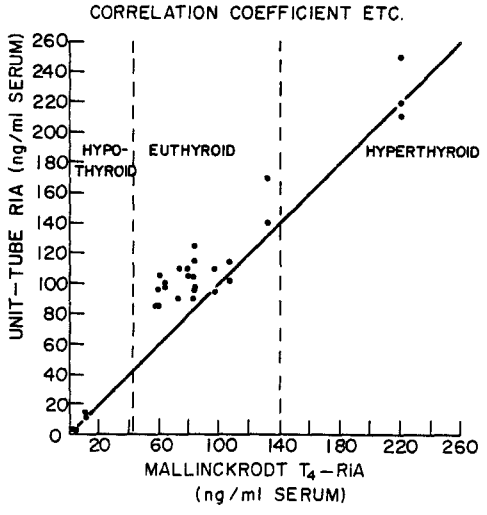

FIG. 2. Correlation with patient samples obtained from a local hospital. The assays were performed with 25 μl of patient serum per tube. Normal T_4 range for the Mallinckrodt assay was found to run from 54 to 130 ng/ml serum.

unlabeled or cold substance. In most cases, the concentration of antibody-combining sites is still in excess when compared with the quantity of label added. The end result is a reduction of incubation time with some possible net gain in sensitivity. Solid-phase immunoassay techniques offer an advantage over classical techniques in that stable antibody complexes saturated with labeled antigen can be prepared. This being the case, we have shown that the introduction of unlabeled antigen results in a displacement of label from the complex without a sacrifice of maximum sensitivity. Another obvious advantage to such a system is that operator error resulting from inaccurate pipetting of labeled material is minimized. Reaction tubes can be prepared to contain all the ingredients necessary for a complete assay with the exception of the sample and standard control serum. This type of procedure reduces an assay (in this case, T_4) to one pipetting procedure. In addition to its simplicity, the assay also offers good sensitivity, stability, and good patient correlation.

The T_4 binding seen with increasing sample size (Fig. 1) has been observed in all solid-phase RIAs so far attempted in our laboratory. The depression of binding appears directly related to protein concentration. Thus, similar results are observed with whole serum and with crystalline bovine serum albumin. Given enough incubation time, the binding eventually reaches the levels observed in buffered systems. Therefore, these observed "nonspecific" protein effects are rate effects due to some factors not yet understood. Because of the phenomenon it is necessary to use a serum sample small enough to diminish the effect it has on the final results. A 25-μl sample works quite well. Even a 50% change in protein concentrate would not cause changes that could be misleading.

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