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Radioimmunoassay of Triiodothyronine (T_3) and Thyroxine (T_4)

An Assay with Both the Bound and Free Fraction of the Hormone Present in the Counting Vessel

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Summary

A radioimmunoassay for triiodothyronine T_3 and thyroxine T_4 without any manual separation is described. By the use of an aqueous two-phase system the free hormone was separated from the immunologically bound fraction and lifted out of the counting area of the gamma counter. The coefficient of correlation between results obtained with this method and with a conventional RIA was 0.97 and 0.93 for T_3 and T_4, respectively.

Index Entries: Radioimmunoassay, of triiodothyronine and thyroxine; aqueous two-phase system, for RIA; triiodothyronine (T_3), RIA of; thyroxine (T_4), RIA of; Partition Affinity Ligand Assay (PALA), of triiodothyronine and thyroxine.

Introduction

Radioimmunoassay (RIA) is a sensitive and widely used method in the assay of drugs, hormones and other substances (*1, 2*).

Competitive binding assays require the separation of free and antibody-bound antigen. This step is often done by hand; it is time-consuming and impairs the accuracy of the assay.

Methods without manual separation are available. One method adsorbs and attenuates the ^{125}I gamma radiation from immunologically unbound triiodothyronine (T_3) with coimmobilized charcoal and bismuth oxide (Bi_2O_3)

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(*3*). In another method a small column containing dry resin is added to a solution containing both free and unbound antigen (*4*). In the example reported unbound digoxin was adsorbed to the dry resins, whereas the antibody-bound fraction migrated up the column and out of the counting area of the gamma counter. Free and bound digoxin and T_3 have also been separated with an aqueous two-phase system (Partition Affinity Ligand Assay, PALA) consisting of $MgSO_4$ and polyethylene glycol. In this assay aliquots were withdrawn from the top phase and counted separately in a gamma counter (*5, 6*).

However, this PALA technique can be used without the final pipeting. After the immunological reaction had taken place, an aqueous two-phase system was added that separated the free from the bound fraction of triiodothyronine (T_3) and thyroxine (T_4). The ratio between the volumes of the phases was chosen so that the top phase containing T_3 or T_4 was outside the counting area of the gamma counter. In this way the activity measured reflected the activity in the bottom phase, which contained the antibody-bound antigen. It was thus possible to assay the hormone without any manual separation.

Materials and Methods

Phadebas® T_3 and T_4 RIA kits and antisera against T_3 and T_4 raised in sheep were generous gifts from Pharmacia Diagnostics AB, Uppsala, Sweden. ^{125}I-labeled T_3 and T_4 and standard sera containing T_3 (0, 0.6, 1.5, 3.0, 8.0, and 16.0 nmol/L), T_4 (0, 6, 20, 60, 150, and 450 nmol/L) were used from the kits.

Bovine serum albumin (BSA) was obtained from Sigma, St. Louis, Mo., USA.

Starch, "soluble" AnalaR and polyethylene glycol 4000 (PEG-4000) were purchased from BDH, Poole, England.

Aliquots of routine human serum samples of T_3 and T_4 were obtained from the hopsital of Lund.

All other chemicals used were of analytical grade.

Test tubes (12 × 75 mm) were purchased from Cerbo, Trollhättan, Sweden.

T_3 and T_4 assay

The assays were routinely performed:

50 μL (T_3) or 10 μL (T_4) serum containing the hormone and 100 μL ^{125}I-T_3 or ^{125}I-T_4 from the kits were mixed with 100 μL of antisera diluted in 0.075 mol/L barbital buffer, pH 8.6, containing 2.5 g/L bovine serum albumin. The samples were incubated for 1 (T_3) or 2 h (T_4).

A well-mixed phase system (3 mL of a 300 g/kg solution of $MgSO_4 \cdot 7\ H_2O$ and 1 mL of a 300 g/kg solution of PEG-4000) was afterwards added to the samples. The samples were allowed 15 min to separate and were then measured in a LKB Minigamma counter (LKB, Bromma, Sweden) without any further separation.

Results and Discussion

Aqueous two-phase systems have been used not only in biochemical preparative work (*7, 8*), but also for analytical purposes (*5, 6, 9*).

If an aqueous two-phase system is used in an analytical system the distribution of the bound and free antigen between the phases must be asymmetric.

T_3 and T_4 are two of the most frequently assayed hormones in clinical chemistry. In a two-phase system consisting of $MgSO_4$ and PEG-4000 practically all the hormones are accumulated in the PEG-rich top-phase. However, if the hormones are bound by antibodies they are held back in the salt-rich lower phase. By performing this separation of free from bound antigen in a test tube and then, without any further separation, measuring it in a gamma counter, the two-phase system lifts the unbound antigen out of the counting area of the gamma counter. This means that the activity measured depends on the amount of bound ^{125}I-labeled antigen (*10*).

In assays for T_3 and T_4 the hormones were incubated with a dilution series of the antisera together with fixed amounts of ^{125}I-labeled T_3 or T_4. After incubation of the T_3 samples for 1 h and the T_4 for two free and bound fractions were separated with a well-mixed two-phase system; 15 min later the samples were measured in a gamma counter with no further separation. Figure 1 shows as an example the dilu-

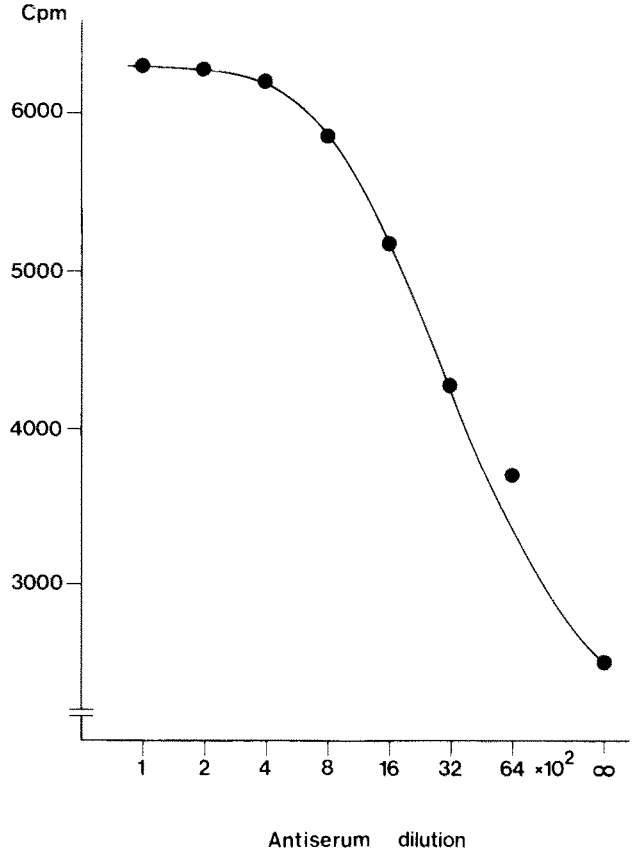

Fig. 1. 100 μL ^{125}I-T_4 and 10 μL T_4 free serum were incubated with 100 μL of a progressive dilution of antiserum. After incubation for 2 h the unbound T_4 was separated with a well-mixed two-phase system, after which the sample was measured in a gamma counter.

tion curve for anti-T_4 serum. As standard dilutions for the assays were chosen antisera dilutions of 1 : 1600 and 1 : 5000 for the T_3 and T_4 assay, respectively.

With these antisera dilutions, calibration curves for T_3 and T_4 were obtained (Figs. 2 and 3). The curves corresponded well to the concentration ranges of clinical interest.

We observed, however, a slight decrease in the measured activity versus time. Both the T_3 and T_4 samples showed such a drift of the counts.

This phenomenon probably depended on a dissociation of the antigen–antibody complex owing to the absence of the free antigen after separation and consequent diffusion of the dissociated antigen out of the counting area up to the PEG-rich phase. The same kind of drift has also been observed in assay systems with Bi_2O_3 as attenuator (*3, 11*).

In an effort to decrease the drift after separation with a two-phase system we tried to reduce the diffusion of the released antigen by adding starch to the two-phase system. The starch passed to the lower phase and had an appreciable effect on the drift (Fig. 4). The mode of action of the starch is not known, but one might

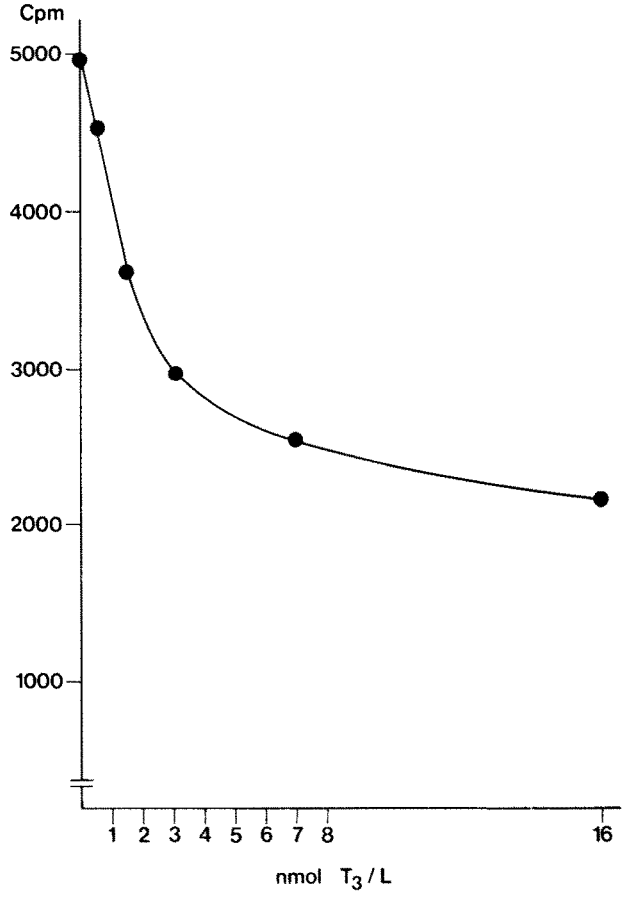

Fig. 2. Calibration curve for T_3: 100 μL ^{125}I-T_3 and 50 μL T_3 standard (0, 0.6, 1.5, 3.0, 8.0, and 16.0 nmol/L) were incubated for 1 h with 100 μL antisera diluted 1 : 1600. The samples were separated and counted as given as in Fig. 1.

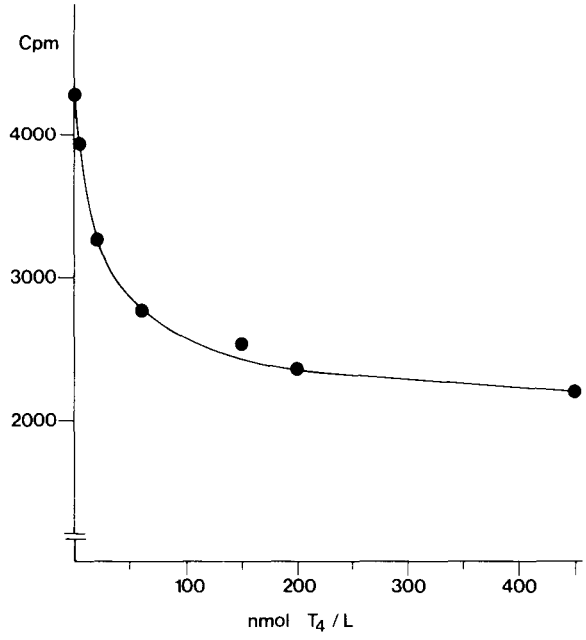

Fig. 3. Calibration curve for T_4: 100 μL ^{125}I-T_4 and 10 μL standard (0, 6, 20, 60, 150, and 450 nmol/L) were incubated for 2 h with 100 μL antisera diluted 1 : 5000. The samples were separated and counted as given in Fig. 1.

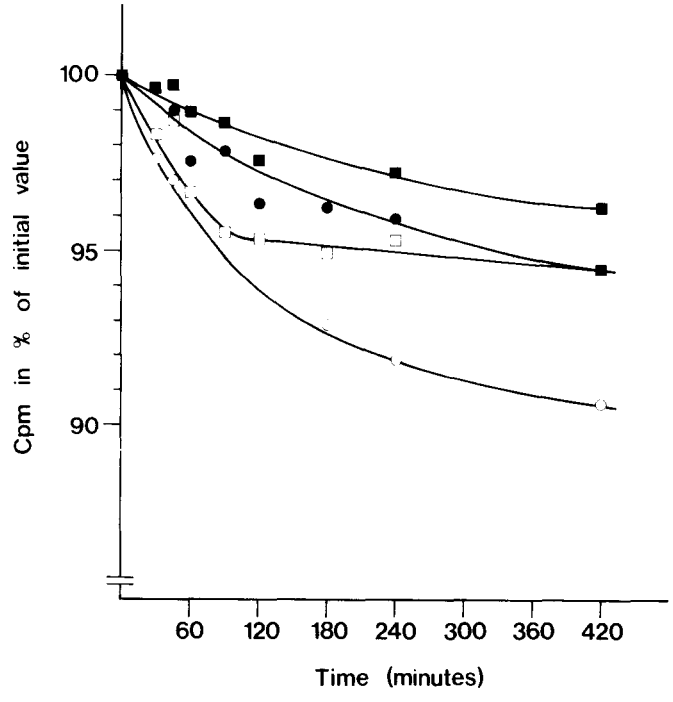

Fig. 4. Measured gamma radiation from a T_3 sample as a function of time: 100 μL ^{125}I-T_3, 50 μL T_3-free serum, and 100 μL antiserum diluted 1 : 1600 were incubated for 1 h. Phase system without any starch (○) and with 0.1 g (□), 0.25 g (●) or 0.5 g (■) starch/4 mL of phase system.

imagine that the starch adsorbs the released antigen or makes the microscopic droplets of PEG persist in the salt phase, i.e., the droplets would otherwise adsorb the released T_3 or T_4 and migrate to the top phase.

T_3 and T_4 sera were measured with both the PALAD-technique and conventional RIA.

When the samples were assayed without any addition of starch to the phase system, the T_3 samples showed a coefficient of correlation of 0.96 ($y = 0.77x - 0.25$) and the T_4 samples (Fig. 5) 0.93 ($y = 0.89x - 14.6$). The slopes of the lines are far from unity because of different standard dilutions were used in the reference and test assay.

However, when the samples were separated with a two-phase system containing 0.5 g starch/4 mL of phase system, the drift was stopped and the coefficient of correlation for the T_3 samples was then 0.97 (Fig. 6, $y = 0.85x + 0.30$).

The use of *P*artition *A*ffinity *L*igand *A*ssay to *D*irect measure the activity from the samples (PALAD) requires no manual separation and the results correspond well to those obtainable by other RIA methods. After addition of starch the system is stable. The PALAD technique should therefore be a useful method, especially in automated assay systems.

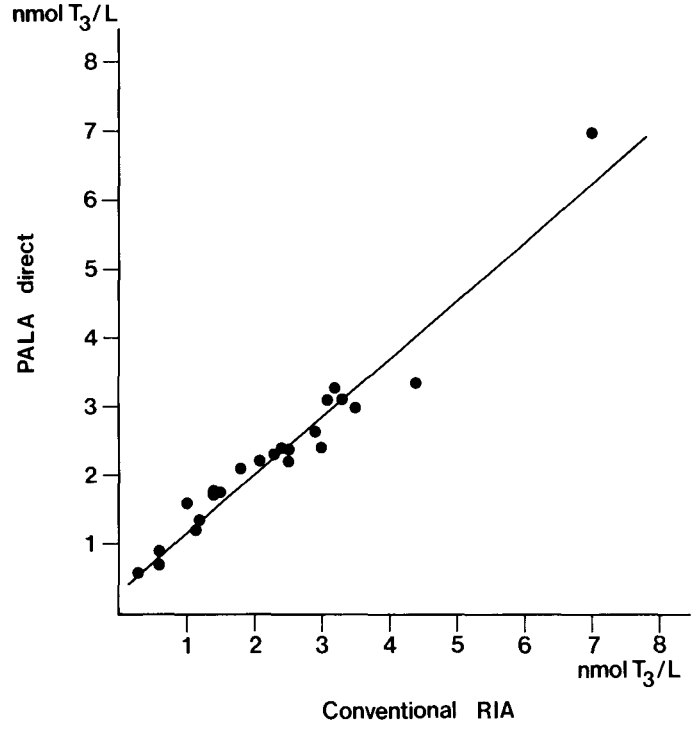

Fig. 5. Correlation between results obtained with a conventional RIA on 23 human serum samples of T_3 and with the PALAD technique. The phase system contained 0.5 g starch/4 mL of phase system.

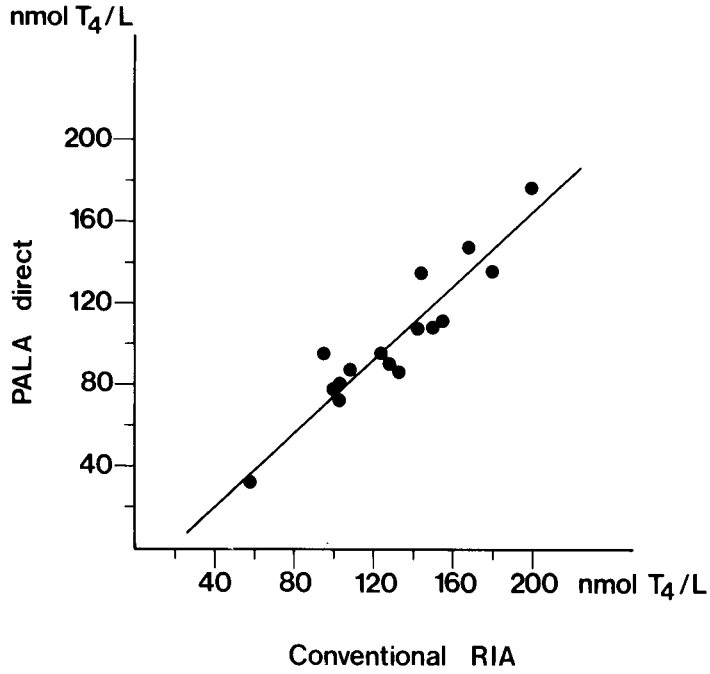

Fig. 6. Correlation between results obtained with a conventional RIA on 16 human serum samples of T_4 and with PALAD technique. Separation with a two-phase system without any starch.

Acknowledgment

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