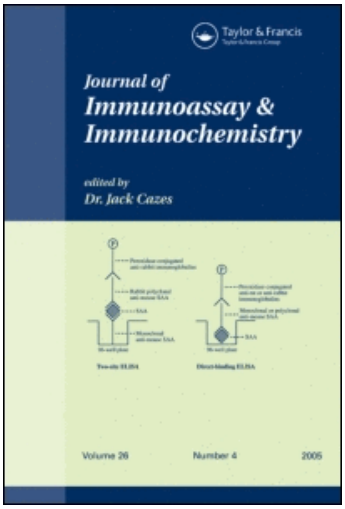

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A SENSITIVE AND SPECIFIC IMMUNORADIOMETRIC ASSAY (IRMA) FOR HUMAN THYROID STIMULATING HORMONE

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ABSTRACT

A liquid phase "two-site" immunoradiometric assay (IRMA) specific for human thyroid stimulating hormone (hTSH) is described. The assay is based on the simultaneous addition of affinity purified sheep anti hTSH IgG-I^{125} and rabbit anti hTSH antiserum to standards and unknowns followed by 4h incubation at room temperature. The separation of free labelled sheep IgG-I^{125} from that bound to hTSH is achieved by the addition of sheep anti-rabbit IgG Fc fragment antiserum. The radiolabelled sheep anti-hTSH IgG-I^{125} was pretreated with solid phase urinary postmenopausal gonadotropins to remove cross reaction with FSH and LH. The assay is specific for hTSH and no cross reaction with the other anterior pituitary glycoproteins or protein hormones has been found. In addition it is characterized by a wide operating range, rapid equilibration of reactants and high sensitivity (0.02 μU/ml). The precision of dose estimates was <10% between 0.25-2.5 μU/ml and <2.5% over the range 2.5-60μU/ml.

INTRODUCTION

The most wide-spread method used so far for the measurement of hTSH is radioimmunoassay (RIA), the major drawbacks of the technique have been ascribed to:

1. existing hTSH antisera commonly cross react with the other glycoprotein hormones.

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2. obligatory use of low anti hTSH antiserum concentrations to achieve adequate sensitivity.

3. instability of radiolabelled hTSH.

4. non-specific serum effects (Hunter and Bennie , 1979).

Even with the most well optimized RIAs, the use of time consuming procedures and very prolonged incubation times are inevitable (Spencer and Nicoloff, 1979; Wehmann et al, 1979; Mewissen et al, 1984).

The introduction of the use of radiolabelled specific antibodies by Miles and Hales (1968) has led to the development of immunoradiometric assays (IRMA) for many analytes in biological fluids. The sandwich-type technique employs two different specific antibodies (one radiolabelled) added in excess and this reduces the time required for the reaction to reach equilibrium, increases the sensitivity and precision, reduces the pipetting error and increases the operating range of the assay. In addition, the increased stability of radiolabelled antibodies has overcome the problem of tracer instability in RIA (Hunter and Budd, 1981).

The limited use of IRMAs in the past has been attributed to difficulties encountered during affinity chromatography, because of the denaturing effects of low pH solutions needed to desorb specific antibodies often resulting in poor recoveries of antibodies, with reduced immunoreactivity (Miles et al, 1974). This has led to the search for high abundance high affinity mouse monoclonal antibodies using the hybridization technique originally described by Kohler and Milstein, 1975.

Recently, however, a new method for the purification of specific, high affinity and highly immunoreactive antibodies from polyclonal antisera has been described (Hodgkinson and Lowry, 1982) which has been used successfully in the development of immunoradiometric assays for a number of human anterior pituitary hormones (Piaditis et al, 1983; Hodgkinson et al, 1984a; Hodgkinson et al, 1984b). In this paper we describe the development of a liquid phase "two-site" immunoradiometric assay (IRMA) for hTSH, using an affinity purified sheep anti-hTSH IgG which after radioiodination is used in conjuction with an unfractionated rabbit anti-hTSH antiserum. For the separation of bound and free labelled antibody a precipitating sheep antibody raised against the Fc fragment of rabbit IgG was used. RIAs were also developed using the purified sheep anti-hTSH IgG and the rabbit antiserum and these have been compared with the hTSH-IRMA.

MATERIALS AND METHODS

Materials

Human TSH (for standards, radiolabelling and preparation of immunoadsorbent), hLH, hFSH and hGH were prepared in this laboratory (McLean et al, 1981). Sheep anti-hTSH (HP/P/1001-IB) was a gift of Guildhay (University of Surrey, Guildford, Surrey) and the rabbit anti hTSH (TR_2B_2) antibody was prepared in this hospital using the immunization schedule described previously (Hodgkinson et al, 1984a). Sheep anti-rabbit Fc precipitating antiserum was prepared by Polyclonal Antibodies Ltd (Blaewaun

Farm, Ffostrasol, Llandysul, Dyfed, Wales). Horse serum 3 (uninactivated) and normal rabbit serum (NRS) were purchased from Wellcome Laboratories Ltd., normal sheep serum (NSS) and rabbit anti sheep precipitating serum from ILS Ltd., (14-15 Newbury Street, London EC1). CNBr-activated Sepharose-4B and Sephacryl S-300 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), chromatography columns for use with solid phases were from Biorad Laboratories,(Watford, Hertfordshire) and for purification of labelled IgGs from Whatman Laboratory Sales Ltd., (Maidstone, Kent, England), absorbent strips from Gelman Ltd., (Ann Arbor, Michigan), Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenyl glycoluril) from Pierce and Warriner (UK) Ltd, β-alanine from BDH chemicals Ltd (Poole, England), postmenopausal urinary gonadotropin (Pergonal) from Serono Laboratories Ltd (UK), bovine serum albumin (BSA) and thiomersal from Sigma Chemical Company.

Methods

Preparation of Immunoglobulin fraction

Antiserum was made 18% (w/v) in Na_2SO_4. After centrifugation (4000g, 30min) and solubilization of the pellet in 0.15M NaCl the IgG was reprecipitated with Na_2SO_4 and the supernatant removed.

Preparation of Solid phases

Human TSH (6mg), Pergonal (750IU hFSH, 750IU hLH) or IgG fraction prepared from 0.5ml of serum was coupled to CNBr-activated Sepharose-4B (0.5g) following the instructions recommended by the manufacturer except that the remaining active sites were blocked

with β-alanine (1%). The non-specifically absorbed materials were removed with successive washes of 0.15M sodium chloride and 0.05M ammonium acetate/0.25M formic acid pH3 buffer containing 20% acetonitrile and 0.01% thiomersal (w/v). Immunoadsorbents were stored at 4°C in saline containing thiomersal (0.02%, w/v) and were washed again as above prior to use.

Preparation of stripped human serum

A human serum pool (50ml) was passed twice through a column packed with the rabbit anti-hTSH IgG (TR_2B_2) immunoadsorbent prepared as above.

Affinity purification of sheep anti hTSH IgG

The specific anti hTSH IgG used for radioiodination in the hTSH-IRMA was purified from the immunoglobulin fraction of 10ml of an anti hTSH sheep antiserum (HP/P/1001-IB). The IgG fraction prepared as above was redissolved in 8ml of 0.15M NaCl. Small samples (10µ l) from this IgG solution and the supernatants from both precipitations were kept for assessment of the progress of the fracionation procedure. Immunoadsorption of sheep IgG on hTSH solid phase was carried out by continuous mixing on an end-over-end mixer overnight at 20°C, followed by gentle centrifugation (500g, 5min). The supernatant was kept for assessment of anti hTSH activity and the gel was packed into a small column and washed, disturbing the bed several times, with 0.15M NaCl (30ml). To each fraction collector tube 200 µl 1M NH_4HCO_3 was added before selective fractional elution of the immunoadsorbed sheep anti-hTSH

IgG. This was achieved by step wise reduction of pH in the following buffers each of which contained acetonitrile (20%, v/v): 0.05M ammonium acetate; 0.05M ammonium acetate/17mM acetic acid, pH5; 0.05M ammonium acetate/66mM formic acid, pH4 and 0.05M ammonium acetate/0.25M formic acid, pH3. The eluting volume of each solution was 9ml.

The IgG content of each eluant fraction (1.5ml + 0.2ml NH_4HCO_3) was estimated by absorbance assuming that A_{280nm}^{1cm} of a 1mg/ml solution is 1.4. Immunoactivity was estimated in the various samples taken during the fractionation of the serum and the eluted anti hTSH IgG fractions by preparing serial dilution curves. Radiolabelled hTSH-I^{125} (200 µl) in 0.05M-PO_4 buffer, pH7.4, containing 0.5% w/v BSA (RIA assay buffer) was added to each antibody dilution (100 µl) again in RIA assay buffer. After incubation for 18h at room temperature, antibody bound hTSH-I^{125} was separated from free hTSH-I^{125} by addition of 50 µl horse serum diluted 1/2 in saline and 600 µl of a polyethylene glycol 6000 solution (20%, w/v in 0.05M phosphate buffer) to each tube, followed by mixing. Centrifugation was carried out immediately (1500g, 8°C, 30min) and the supernatant aspirated to waste. The precipitate was counted in an Innotron Hydragamma 16 counter. The peaks of IgG observed after each pH step (pH5, pH4, and pH3) were pooled separately and dilution curves were prepared with each IgG pool as described above. The dilutions of antibody which bound 50% of added hTSH-I^{125} were chosen for the development of comparative hTSH radioimmunoassay standard curves which enabled

the affinity constant of each IgG pool to be determined by Scatchard analysis (Scatchard, 1949).

Iodination of sheep anti hTSH IgG

The sheep IgG-I^{125} tracer was prepared using a modification of the iodogen method (Salacinski et al, 1981). Iodogen (1mg) was dissolved in dichloromethane (25ml) and 100 µl of this solution was dispensed into a polypropylene iodination vial and the solvent evaporated to dryness at room temperature. Phosphate buffer (30 µl, 0.5M, pH7.4) was added to the vial prior to iodination, followed by NaI^{125} (5 µl, 0.5mCi, 0.25nmole) and sheep anti-TSH IgG (50 µl, 0.125nmole). The iodination was allowed to proceed for 10min and was terminated by transferring the vial contents to another vial containing 0.05M phosphate buffer (250 µl, pH7.4). The mixture was left standing for a further 10 min at room temperature followed by addition of normal sheep serum (250 µl) as a carrier. This mixture was then loaded onto a Sephacryl-300 column. The peak of radioactivity eluted in the expected position for sheep IgG (Kav = 0.26) was pooled followed by the addition of normal sheep serum to a final concentration of 10% v/v. The incorporation of radio-iodine in the IgG was normally between 70 and 90% and the specific activity of the tracer ranged from 9-15µ Ci/µg as estimated by the absorbent strip method (Orskov, 1967).

Treatment of labelled IgG with Pergonal solid phase

In order to eliminate any cross reaction of the sheep anti-TSH IgG-I^{125} with the FSH and LH, it was passed twice through a small

column packed with the Pergonal solid phase. The eluted IgG-I^{125} was diluted to 10ml with phosphate buffer 0.05M, pH7.4, containing normal sheep serum (10%, v/v) and mannitol (1%, w/v) prior to storage in 0.5ml samples at -20°C.

Preparation of thyrotropin standards

Thyrotropin prepared in this laboratory was calibrated against the WHO TSH (68/38) in order to prepare the working standards. This was diluted in normal horse serum (previously filtered through a 0.8 μm filter) to give a range of standards (0.2-100 μU/ml) which were stored at -70°C.

Development of TSH-IRMA assays

The effect of simultaneous and separate addition of sheep-anti TSH IgI^{125} and rabbit anti hTSH antiserum on the performance characteristics of the TSH-IRMA was assessed as follows:

A. Simultaneous addition.

To duplicate hTSH standards (200 μl) a reagent mixture (200 μl) was added containing the sheep anti hTSH IgG-I^{125} (80,000cpm) and rabbit anti hTSH antiserum mixture (1/80,000) in IRMA assay buffer (0.05M phosphate buffer, pH7.4, containing 0.5% BSA, w/v, 2% PEG 6000, w/v, 1% NRS, v/v, 2% NSS, v/v) followed by incubation (4h, at room temperature).

B. Delayed addition of rabbit anti hTSH antiserum.

To duplicate hTSH standards (200 μl), sheep anti hTSH IgG-I^{125} (80,000cpm/100 μl, in IRMA assay buffer) was added, the tubes vortexed and incubated for 2h at room temperature prior to the

addition of rabbit anti hTSH antiserum (100 µl, 1/80,000, in IRMA assay buffer) followed by a further 2h incubation at room temperature.

Precipitation system

Separation of bound and free labelled antibody was achieved using a sheep anti rabbit IgG Fc fragment serum solution (200 µl, diluted 1/20 in 0.05M phosphate buffer, pH7.4) in the presence of normal rabbit serum (1%, w/v) as carrier (Buckler, 1971) which had been previously added to the IRMA assay buffer. After incubation (room temperature, 1/2h) 1ml of PEG (2%, w/v in 0.05M PO_4 buffer pH 7.4) was added to each assay tube followed by centrifugation (2000g, 30min, 8°C) and aspiration of the supernatants to waste. The final assay procedure was as described in A. The precision of the assay between 0 and 0.2 µU/ml could be improved however if the precipitate was washed with an extra 1ml of the PEG-6000 solution followed by re-centrifugation and aspiration of supernatant to waste. The radioactivity of the precipitates was counted for 1min in the Innotron Hydragamma-16 counter coupled to an Apple IIe computer using the curve-fitting logit-log transformation in which four-parameters statistics were fitted (intercept, slope, 50% dose, correlation).

Radioimmunoassays for h-TSH

A sensitive radioimmunoassay to h-TSH (RIA-S) was developed according to the method described by Chard (1978) using the affinity purified pH3 pool sheep anti-h-TSH IgG as the antibody.

The assay procedure was as follows: RIA assay buffer (200 µl) and sheep anti hTSH IgG (50 µl, 1/10,000 solution in RIA assay buffer) were added to duplicate hTSH standards 200 µl). The mixture was vortexed and incubated for 18h at room temperature prior to the addition of hTSH-I^{125} (50 µl, 4,000cpm in assay buffer) and a further incubation for 18h at room temperature. Precipitation of bound hTSH was achieved by adding a rabbit anti-sheep IgG antiserum (200 µl, diluted 1/20 in 0.5M phosphate buffer pH7.4). After incubation (1/2h, room temperature), each tube was centrifuged (2400g, 1/2h, 4°C) and the supernatants aspirated to waste. A radioimmunoassay was also developed using the rabbit antibody TR_2B_2 (RIA-R). The assay format was essentially the same as above except that it was optimized for a greater range using 20,000cpm h-TSH; 1% normal rabbit serum was used as a carrier and sheep anti rabbit precipitating antibody for separation.

RESULTS AND DISCUSSION

Progress of sheep IgG fractionation

Over 90% of the initial sheep anti hTSH activity, as assessed by antibody dilution curves, was found in the final redissolved Na_2SO_4 precipitate. After solid phase adsorbtion <5% was found in the supernatant.

Selective fractional elution of sheep anti hTSH immunoglobulins

The selective elution profile of sheep anti hTSH IgG is shown in Figure 1. The bulk of sheep anti hTSH IgG (571 µg) (as assessed by extinction at 280nm and by preparation of antiserum dilution

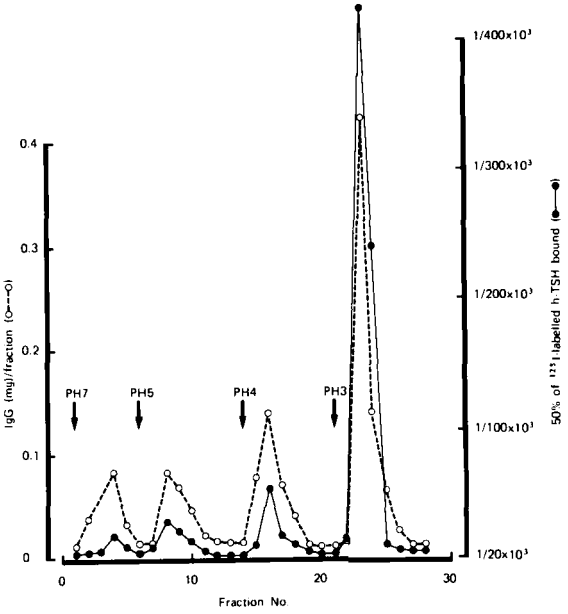

FIGURE 1 Selective elution of sheep anti-hTSH IgG by stepwise reduction of pH. The affinity constant of the pooled sheep IgG eluted at pH3 (fractions 23-25) was 2.14 x 10^{11} l/mol. The affinity constant of pooled sheep IgG eluting at pH5 (fractions 8-10) was Ka= 1.0 x 10^{11} l/mol) and at pH4 (fractions 15-17) was Ka= 1.1 x 10^{11} l/mol).

curves) eluted at pH3 (fractions 23,24,25) and the rest at pH4 (fractions 15-17), pH5 (fractions 8-10) and pH7 (fractions 3-5). Fractions eluting after each pH step were pooled separately and antiserum dilution curves prepared. The dilution of each pool which bound 50% of h-TSH-I^{125} was used for the preparation of RIA standard curves in order to estimate their respective affinity constants by Scatchard analysis. Immunoglobulins with the highest affinity (Ka=2.14 x 10^{11} l/mol) were eluted at pH3 while IgG which eluted at pH5 and pH4 represented populations of antibodies with lower affinities (Ka pH5 = 1 x 10^{11} l/mol, Ka pH4 = 1.1 x 10^{11} l/mol). Acetonitrile was removed under a stream of nitrogen and 50 µl (125pmol) samples were stored at -70°C for radioiodination.

Radio-iodination of sheep anti hTSH immunoglobulin

The radiolabelled sheep anti hTSH IgG-I^{125} after chromatography on Sephacryl S-300 eluted as a single homogeneous peak of radioactivity in the expected position for sheep IgG (Kav = 0.26). The incorporation of ^{125}I into the sheep IgG ranged between 70-90% and the specific activity was 9-15 µ Ci/ µg. The tracer was stable retaining high levels of immunoreactivity losing only 31% of its initial immunoreactivity after storage for 2 months. Attempts to increase the specific activity did not result in any improvement of assay sensitivity or performance but led to reduced stability of the immunoglobulin.

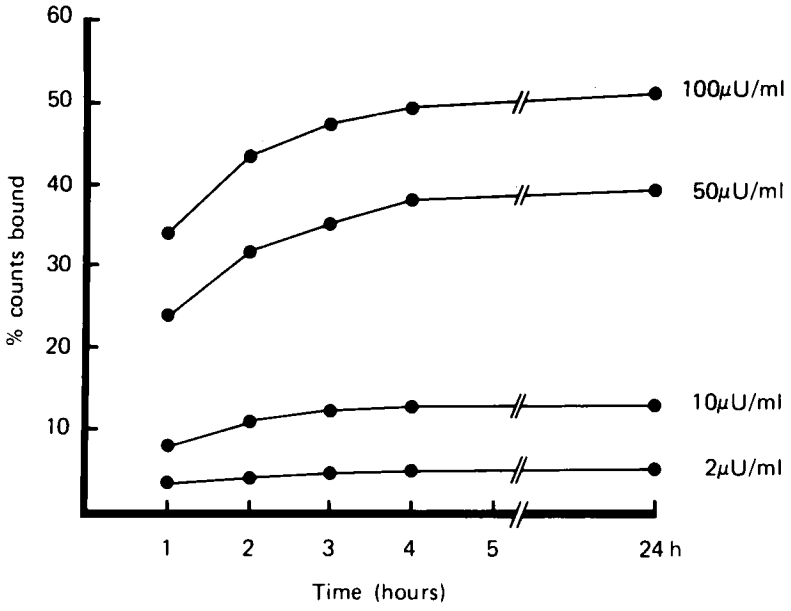

FIGURE 2 Rate of equilibration of the hTSH-IRMA at room temperature.

Performance characteristics of the assay

As shown in Figure 2, the equilibration of the binding reaction (sheep IgG-I^{125}, 80,000cpm and rabbit antiserum, 1/80,000) was >90% completed after 4h incubation, at room temperataure and no further significant improvement of assay sensitivity was attained with more prolonged incubation times.

An important factor affecting the working range and sensitivity of the assay was the final rabbit hTSH antiserum concentration. Progressively decreasing the rabbit antiserum concentration (1/20,000-1/80,000) was accompanied by an increase of precipitated bound-hTSH fraction resulting in a progressive improvement of the working range and sensitivity of the assay (results not shown).

This was suggestive of an element of competition for the same antigenic epitopes on the hTSH molecule at high concentrations of the rabbit antiserum. Maximal precipitation (>70%) was attained with 200 μU/ml of h-TSH when the rabbit antiserum was at a dilution of 1/80,000 and the sheep IgG-I^{125} radioactivity was 80,000cpm. Varying the amount of sheep IgG-I^{125} from 75,000-150,000cpm/tube had little effect on the sensitivity of the assay. A further increase of rabbit anti hTSH antiserum dilution (>1/120,000) in the reagent mixture resulted in a high dose "hooking" effect.

Interestingly delayed addition of rabbit antiserum had little effect on performance characteristics of hTSH-IRMA. This can be explained by minimal competition observed between the antibodies at the concentration employed for reagent mixture preparation and the high affinity of the sheep anti hTSH IgG.

Normal horse serum as a diluent for TSH standard curve

Two TSH-IRMA standard curves (range: 0-100 μU hTSH/ml) were prepared using as diluent normal horse serum and a pool of TSH stripped human serum. An additional curve was prepared by successive dilutions of a hypothyroid serum of known value using normal horse serum as diluent. All three standard curves were superimposable (results not shown).

Specificity

Since there is a high degree of sequence homology between the pituitary glycoprotein hormones an investigation was undertaken to

determine the interference from the gonadotropins in the TSH IRMA. Preliminary studies indicated a slight rise in "apparent" TSH in the serum of three patients following the i.v. administration 100ng LHRH, measured with TSH IRMA (Fig. 3A). The problem was eliminated by using sheep anti-hTSH IgG-I^{125} which had been passed through a small column packed with Pergonal solid phase before preparation of the reagent mixture (Fig. 3B). No cross reaction with HCG and hGH was found at concentrations 1000ng/ml and 2000ng/ml respectively.

Comparison of hTSH-IRMA with hTSH-RIAs

Figure 4 shows the comparative precision profiles (Ekins, 1981) of the "two-site" liquid phase hTSH-IRMA and of the two hTSH-RIAs using the same two antibodies. Sixty four samples for routine clinical assay were measured in each of the above assays. The precision potential of dose estimates for hTSH-IRMA was <2.5% over the range 2.5-60 μU/ml and <10% between 0.25-2.5 μU/ml and 60-200 μU/ml. For RIA-S it was <5% over the range 2-18 μU/ml, <28% between 0.5-2 μU/ml and <10% between 18-50 μU/ml and for RIA-R it was <10% over the range 4-74 μU/ml and <44% between 0.7-4 μU/ml. In contrast to TSH-RIA, TSH-IRMA was characterized by a wide operating range and high sensitivity. This is very difficult to achieve with any RIA unless the anti hTSH antiserum has high affinity and the assay development follows copious and time consuming procedures (Spencer and Nicoloff, 1980; Mewissen , Lippe , Dudek , Hehrmann , 1984). The low non-specific precipitation of labelled anti hTSH IgG-I^{125} by constituents of individual serum

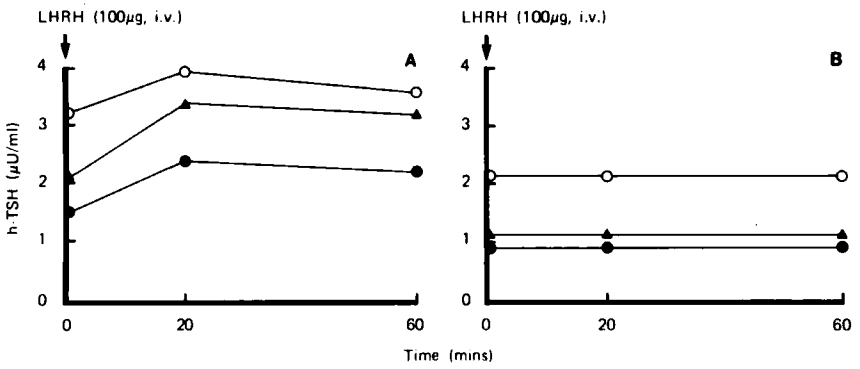

FIGURE 3 The affect of LH-RH on apparent "hTSH" concentrations as estimated by the IRMA. "h-TSH" levels measured in the serum of three patients after i.v. administration of LH-RH (100 μg) using (A) untreated IgG-I^{125} and (B) pergonal pretreated IgG-I^{125} in the preparation of reaction antibody mixture.

samples and normal horse serum gives the TSH-IRMA a remarkably higher precision than the TSH-RIAs.

TSH-IRMA standard curves (Figure 5) are characterized by a 41 fold change in bound radioactivity across the range of standards. In contrast a 15 and 7 fold change in bound radioactivity was found in RIA-S and RIA-R respectively and a reduced operating range was observed in both of them. The sensitivity of the IRMA was 0.02 μU/ml, as defined by a 2.5 standard deviation increase of duplicate counts from zero hTSH, while in RIA-S it was 0.6 μU/ml and in RIA-R 1.0 μU/ml. The relatively wide operating range of RIA-R was achieved at the expense of sensitivity of the assay while the high sensitivity of RIA-S compared to RIA-R is explained by the high affinity of sheep anti hTSH IgG. The between assay precision of the TSH-IRMA was 4.5% as calculated for TSH measurements in 15 different human samples in 10 different assays at the level of 1.54 ± 0.07 μU/ml.(mean; SD).

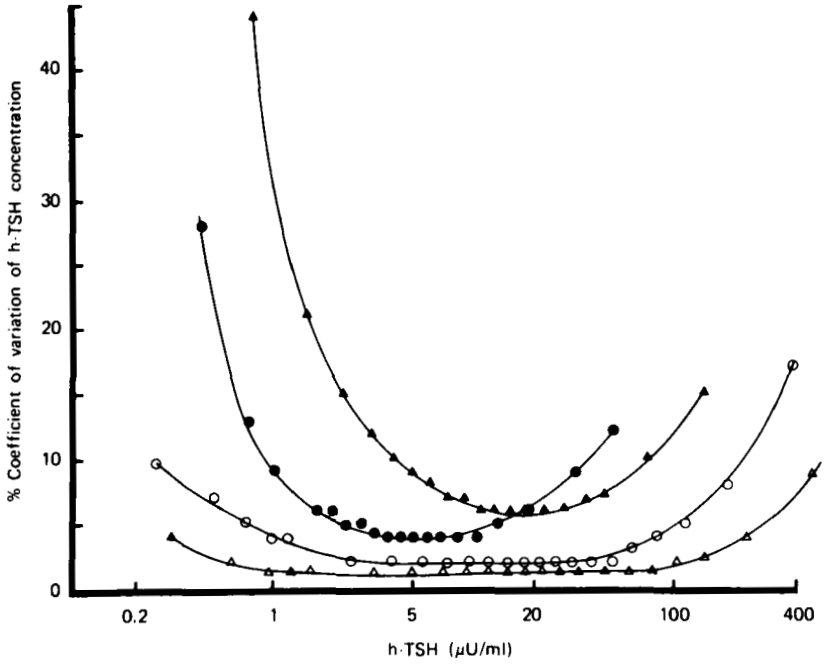

FIGURE 4 Comparative precision profiles of the hTSH-IRMA, before (o-o) and after (△-△) washing with 1ml, 2% PEG 6000 and two hTSH-RIA's. RIA-S (●-●) employed the affinity purified sheep anti hTSH IgG and RIA-R (▲-▲) the rabbit (TR_2B_2) anti hTSH antiserum.

There are a number of TSH-IRMAs commercially available which employ monoclonal antibodies raised either against the intact hTSH molecule or the α and β subunits separately. In most, one of the specific antibodies is bound to solid phase to separate the TSH bound IgG-I^{125}. These assays are characterized by inconvenient continuous mixing of reagents at 37°C and successive washes to reduce the non-specific binding of labelled IgG-I^{125}, with less sensitivity than the "two site" liquid phase TSH-IRMA described in this paper. In addition one of these assays which employs monoclonal antibodies raised against the α and β hTSH

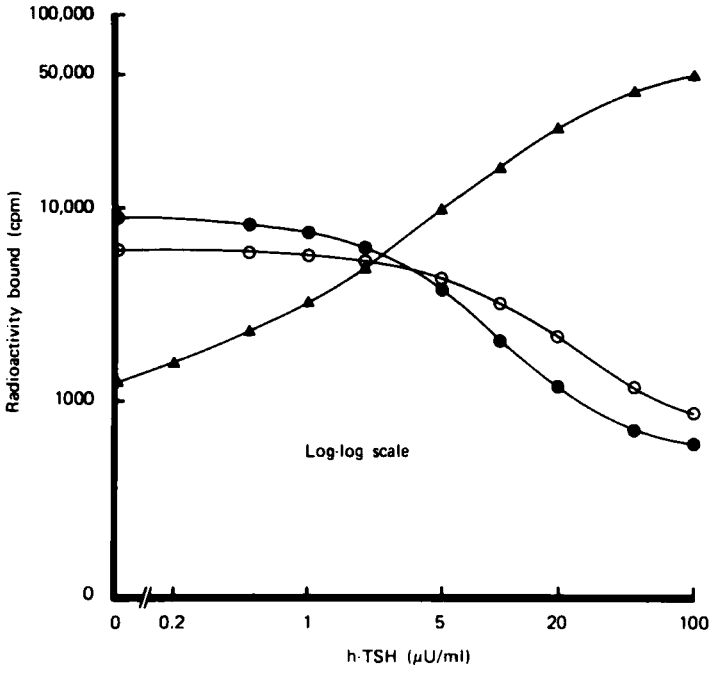

FIGURE 5 Comparative hTSH-IRMA and two hTSH-RIAs standard curves showing changes in bound radioactivity across the range of standards in RIA-S (●-●) and RIA-R (o-o) respectively compared with the hTSH-IRMA (▲-▲).

subunits separately cannot avoid some cross reaction with FSH and LH (Pekary and Hershman, 1984). In contrast the TSH-IRMA described here is specific for hTSH, is simple to perform requiring only simultaneous addition of sheep and rabbit anti TSH antibodies to standards and unknowns followed by a 4h incubation at room temperature. The addition of a sheep anti-rabbit Fc antibody precipitates the TSH bound fraction. The high sensitivity and specificity of the assay should allow us to distinguish hyperthyroid patients from normals with a single basal

TSH estimation and reduce the need for the TRH stimulation test. This work will be the subject of a further report.

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Reprints requests to Dr. G. Piaditis.

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