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EVALUATION OF A COMMERCIAL REAGENT SYSTEM FOR THE RADIOIMMUNOASSAY OF HUMAN THYROID STIMULATING HORMONE

by
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B.A., Queen's University, 1973

THESIS

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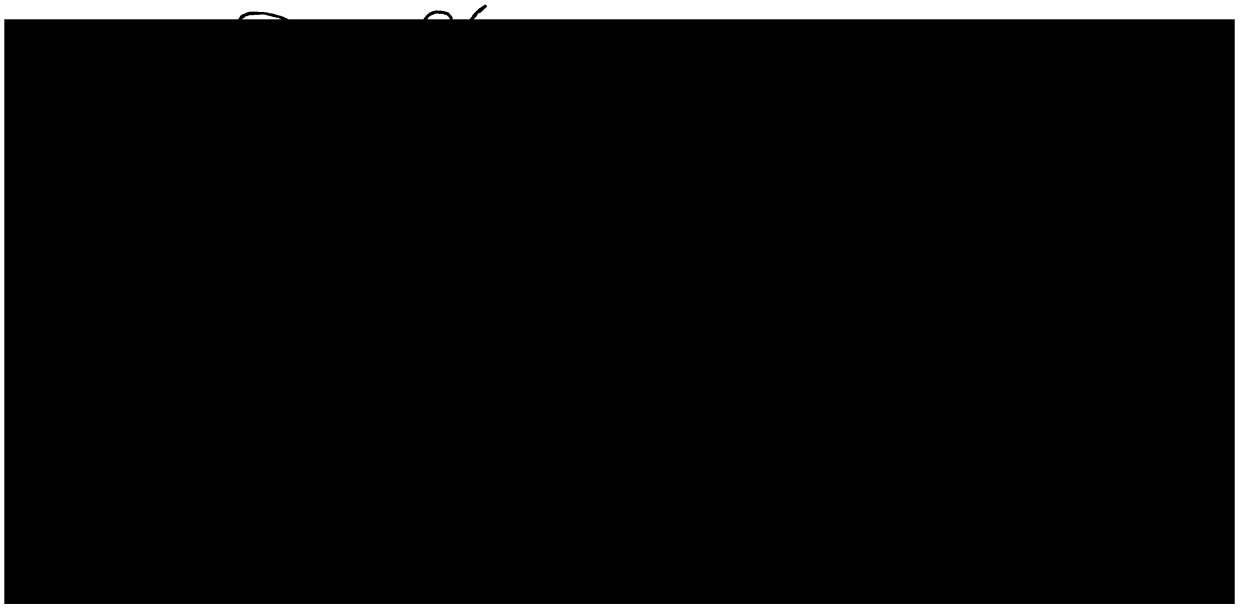

I N D E X

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INTRODUCTION

Measurement of serum thyrotropin (TSH) is of increasing clinical interest. Immunoassay of TSH is now recognized as a sensitive diagnostic parameter in the diagnosis of hypothyroidism. It is also of clinical value in the monitoring of thyroid replacement therapy (1,2,3). Thyrotropin, secreted by the anterior pituitary body is a carbohydrate - containing polypeptide hormone with a molecular weight of 26000-30000(4). The regulation of thyroid function by this hormone constitutes an example of classic feedback theory (see Figure 1). TSH induces the production and release of thyroxine and triiodothyronine from the thyroid gland. An increase in the plasma content of these hormones inhibits the secretion of TSH. This process is partly controlled by the hypothalamus and the central nervous system. Following stress or the action of an external stimulus, the anterior hypothalamus secretes the peptide, "thyrotropin releasing hormone" (TRH), which stimulates the secretion of TSH from the pituitary. Recent studies report the secretory effect of TRH is blocked by triiodothyronine and thyroxine, the latter being more active than the former (5). Although the hypothalamus is thyroxine - sensitive, the anterior pituitary is the main site of feedback inhibition by the thyroid hormones. The stimulatory affect of TRH is blocked by increased levels of circulating thyroid hormones acting directly on the pituitary.

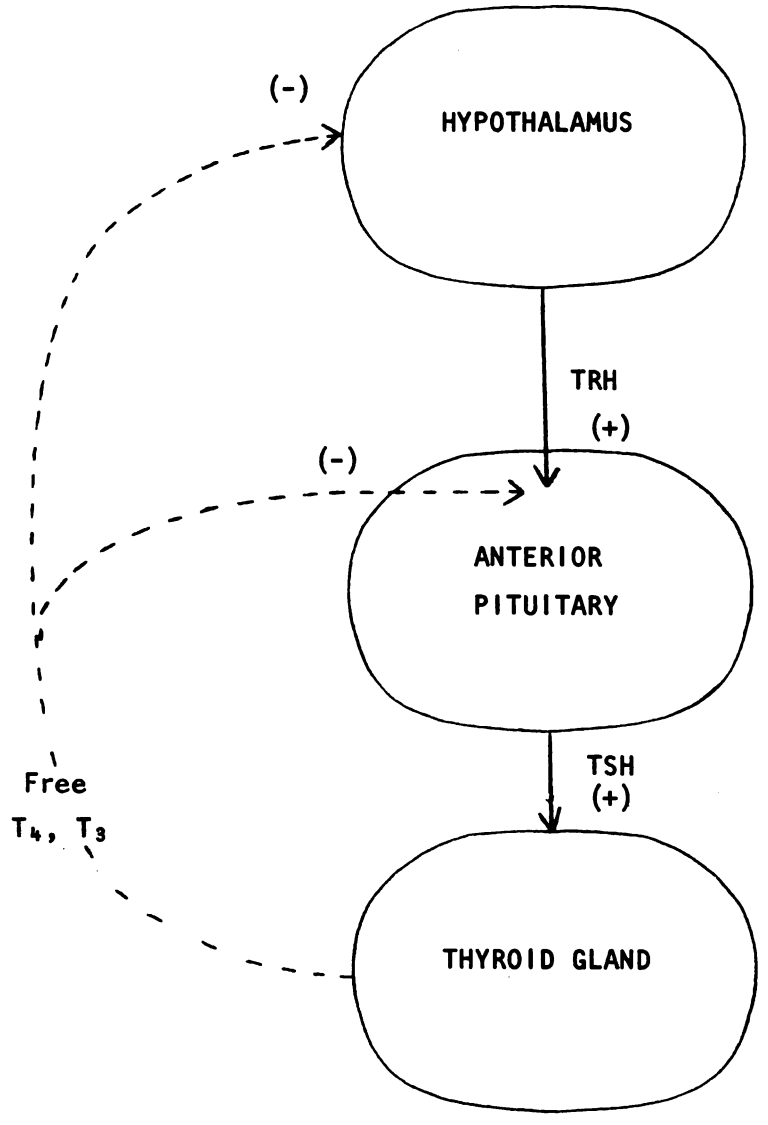


Figure 1: DIAGRAM OF THE NEGATIVE FEEDBACK MECHANISM REGULATING THYROID FUNCTION. Solid Arrows signify stimulation; dashed arrows, inhibition. TRH, thyrotropin releasing factor; TSH, thyroid stimulating hormone; T_3, triiodothyronine; T_4, thyroxine

CLINICAL SIGNIFICANCE OF SERUM HTSH

In disease states, the serum TSH levels reflect disturbances in the hypothalamic-pituitary-thyroid axis. The assay of serum thyroid stimulating hormone is particularly useful in the diagnosis of primary hypothyroidism. The levels in primary hypothyroidism are markedly elevated, even when the serum thyroxine (T_4) is normal. The presence of a low serum thyroxine with an elevated TSH value is indicative of primary thyroid disease. Patients with secondary hypothyroidism have low or absent levels of TSH, reflecting myxedema of the pituitary or hypothalamus, and an impaired responsiveness to depressed plasma levels of thyroxine, and triiodothyronine. Thus serum TSH levels can be diagnostic in distinguishing primary from secondary hypothyroidism (6). In diffuse hyperthyroidism (Graves' Disease), low levels of TSH have been reported, supporting the concept that normal TSH regulatory interrelationships still apply, and the thyroidal activity is maintained by some abnormal thyroid-stimulating factor (6). In hyperthyroidism associated with autonomous hyperfunctioning nodules, TSH levels are low while plasma levels in thyroiditis are variable depending on the levels of circulating thyroid hormones. A significant percentage of patients suffering chronic thyroiditis are found to have elevated TSH values, although their thyroid function tests may be within the euthyroid range (6). A summary of test results found in thyroid disease is presented in Table 1.

The recent introduction of synthetic thyrotropin releasing hormone, a potent stimulator of TSH release, further contributes to the clinical potential of serum TSH measurements. A standard intravenous TRH test has been developed to aid in the diagnosis of even minor disturbances in the negative feedback mechanism (7).

TABLE 1: SUMMARY OF TEST RESULTS IN THYROID DISEASES (5,8)

THYROID DISEASES:	T_4	RESIN T_3 UPTAKE	FREE THYROXINE INDEX	T_3 RIA	TSH
HYPERTHYROIDISM	↑	↑	↑	↑	↓
T_3 THYROTOXICOSIS	N	N	N	↑	↓
HYPERTHYROIDISM IN RELAPSE	N ↑	N ↑	N ↑	↑	N or ↓
PRIMARY HYPOTHYROIDISM	↓ N	↓ N	↓ N	-	↑
SECONDARY HYPOTHYROIDISM	↓	↓	↓	-	N ↓
DECREASED THYROID RESERVE	N	N	N	-	Borderline to ↑
THYROIDITIS	VAR	VAR	VAR	VAR	VAR; often ↑
PREGNANCY BIRTH CONTROL PILLS ESTROGENS	↑	↓	N	↑	↓
NEPHROSIS SEVERE LIVER DISEASE	↓	↑	N	-	N
REPLACEMENT THERAPY:					
L-THYROXINE (SYNTHYROID)	N	N	N	N	N
TRIIODTHYRONINE (CYTOMEL)	↓	N ↓	N ↓	N	N
DESSICATED THYROID	N ↓	N ↓	N ↓	N	N

↑ = Increased

↓ = Decreased

VAR = May be Decreased, Normal, or Increased

N = Normal

Serum TSH measurements at twenty and sixty minutes follow the intravenous administration of TRH. In normal individuals, the serum TSH rises from its basal level to reach a peak around twenty minutes before falling again. In hyperthyroidism the TSH response is impaired, even when other thyroid function tests are normal or equivocal such as in thyrotoxicosis (7). Primary hypothyroidism is reflected in an excessive TSH response from a normal or elevated basal level. According to Besser and Mortimer (7), the condition is not primary hypothyroidism unless an excessive response is found. Secondary (pituitary) hypothyroidism is usually associated with impaired twenty minute and sixty minute responses, whereas a delayed response is characteristic of hypothalamic hypothyroidism, with the sixty minute sample being higher than the twenty minute specimen (7). Thus the standard intravenous TRH test makes it possible to differentiate between hyperthyroid patients and the normal group. Similarily hypothyroids can be diagnosed, including hypothalamic hypothyroidism a condition which may not be uncommon (9). It is also a useful test in cases of suspected thyrotoxicosis when serum T_4 and resin uptake tests are normal (7, 9). A summary of expected TSH responses is presented in the recent review article offered by Besser and Mortimer (7).

Until recently, TSH measurements have been limited to research and to special diagnostic problem cases. Bioassay methods are complex and expensive. Radioimmunoassays have also been complex requiring the preparation and purification of a labelled antigen, and the production and standardization of a specific antisera. Published procedures have been time consuming, with incubation periods ranging up to 5 days (4,9,10). A number of problems associated with TSH immunoassay have been reported (9,11,12). The measurement of serum TSH in the diagnostic clinical laboratory has therefore been impractical. With the recent

proliferation of commercial radioimmunoassay kits and reagents, a number of systems and components have become available for the assay of human thyroid stimulating hormone (HTSH). However, before a commercial reagent system is implemented in the clinical laboratory it is important that it be evaluated carefully and thoroughly (13,14,15). A good reagent system manufactured with stringent quality control can offer the clinical laboratory a convenient means of providing useful laboratory data which may aid in the early diagnosis of disease. In contrast, a poor system may provide inaccurate and misleading data which could be hazardous to the patients well-being. To be useful in the clinical diagnostic laboratory, an assay system must be simple, quick to perform, and reliable. It must offer the level of accuracy, precision, sensitivity, and specificity required to meet the clinical need. The "Beckman Twin 10-Test Reagent System," [1] a commercial system available for the quantitative radioimmunoassay of human thyroid stimulating hormone (HTSH), will be evaluated for use in the clinical diagnostic laboratory. A review of the basic principles and theory of radioimmunoassay technique and design will be presented and subsequently applied in the evaluation of the Beckman HTSH System. The reagent system and the suggested protocol will be characterized through an experimental evaluation of the reaction sequence, the assay of quality control and patient sera, and the evaluation of dose-response curves. The accuracy, precision, sensitivity, and specificity, of the Beckman system will be evaluated. The results of reproducibility studies, recovery experiments, and tests of specificity will be reported. Low, normal and elevated plasma TSH levels assayed using the Beckman HTSH Reagent System will be compared to reference laboratory values obtained using a solid-phase antibody method. A study correlating serum T_4,

[1] Beckman Instruments Inc., Fullerton, California

T_3 uptake, and free thyroxine index (FTI), with TSH levels in patient sera analyzed by the Beckman kit will be offered. The general reliability, and practicability of the Beckman Twin 10-Test Reagent System will be discussed, and recommendations made to optimize the use of this system in the clinical laboratory. A detailed description of the method, equipment, and reagents employed will be included.

GENERAL PRINCIPLES AND THEORY OF RADIOIMMUNOASSAY

General Principles

Unknown concentrations of an antigen may be determined by allowing radiolabelled antigen molecules (Ag*) and nonlabelled antigen molecules (Ag), to compete physiochemically for the binding sites of antibodies (Ab). Under optimal conditions, increasing amounts of the unlabelled antigen (Ag) progressively saturate the limited binding sites of the antibody (Ab), allowing less of the radiolabelled antigen (Ag*) to be bound. Following incubation of the three essential components (Ag, Ag*, Ab), the antigen-antibody complexes or "bound antigens" are physically separated from the free antigens and the radioactivity of either or both phases is measured (16). A number of different procedures have been employed in the separation of bound and free antigens. The Beckman HTSH System uses a "double antibody" technique in which a second antibody (AbII) complexes with the primary antibody (Ab) precipitating the antigen-antibody complexes. Figure 2 shows the generally accepted concept of antigen-antibody interaction in radioimmunoassay. Thus an unknown amount of unlabelled hormone (Ag) may be quantitated by comparing its distribution between "bound and free," with the distribution yielded by a set of standards containing known concentrations of the hormone. The distribution of radioactivity may be expressed in terms of one of a number of response metameters such as the ratio of the two fractions (free/bound or bound/free), or as a percent of the total counts bound (%B). Percent bound can then be plotted as a function of the concentration of unlabelled hormone (Ag) in the system, (see Figure 3).

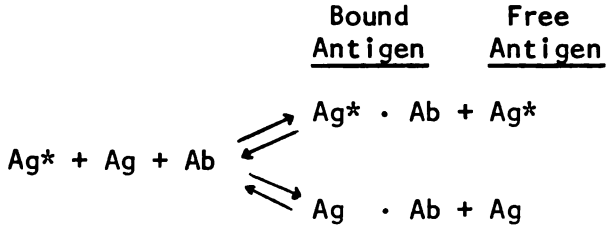


Figure 2: Diagram showing antigen-antibody interaction as generally conceived for radioimmunoassays.

Ag* radiolabelled antigen
Ag nonlabelled antigen
Ab antibody

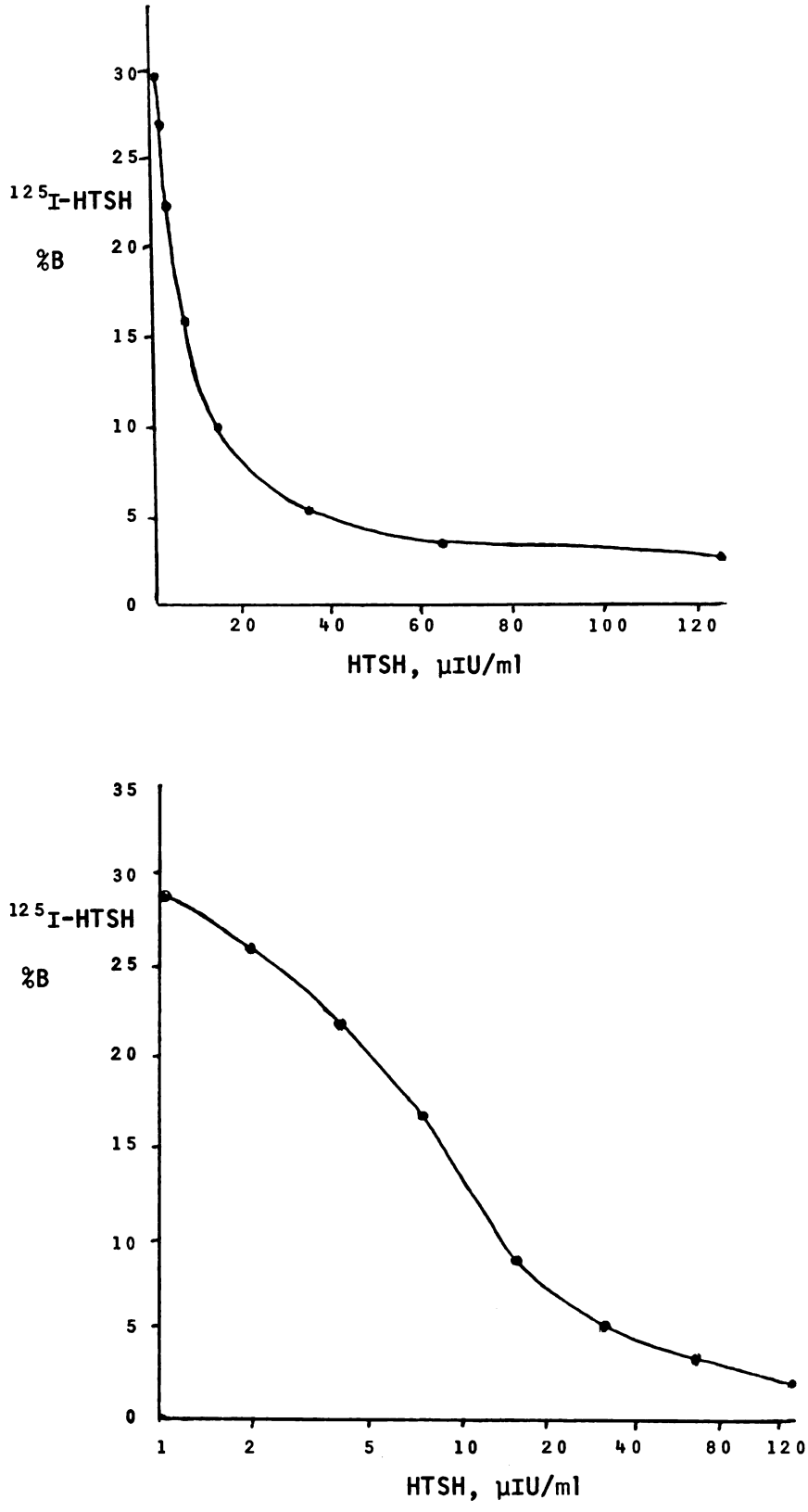


Figure 3: Dose-response curves obtained using the Beckman HTSH System.
Upper Curve: plotting %B versus HTSH concentration
Lower Curve: plotting %B versus log HTSH concentration

As presented in Figure 2, the generally accepted concept recognizes that antigen-antibody interactions are reversible. In practice, the radioimmunoassay technique used most frequently, allow the competition between labelled and unlabelled antigen to occur simultaneously until a mass equilibrium is reached between the antigen and antibody. This technique has been referred to as "equilibrium saturation" (17). It has been found however, that increased sensitivity can sometimes be gained by saturating the antibody in two successive steps (18). The first step involves the incubation of the unlabelled antigen with the antibody. The labelled antigen is then added to the incubation mixture in step two. To eliminate or diminish the simultaneous competition between labelled and unlabelled antigens for the antibody, the reaction is stopped before equilibrium is reached. This technique is referred to as nonequilibrium analysis or "sequential saturation" (18). Zettner has discussed the principles, and differences, between equilibrium and sequential saturation techniques in two recent articles (17, 18).

In the equilibrium method, Ag is first mixed with a fixed amount of Ag*. The antibody Ab, is then added and the mixture incubated to reach an equilibrium state between the reactants. Thus during their simultaneous incubation, Ag* competes with Ag for the available binding sites of Ab. In sequential saturation such competition is eliminated or at least greatly reduced. Ag is first mixed with an amount of Ab whose binding capacity exceeds the total amount of Ag added. Essentially all of the Ag can become bound at equilibrium during the first incubation period. In the second step, Ag* is added to react with the binding sites that have remained unoccupied by Ag during the initial incubation. Competition is thus confined to the second incubation step. The potential increase in sensitivity by sequential saturation derives from the fact

that the reaction at the end of incubation two is terminated before a full exchange of Ag* for Ag occurs. Less Ag* · Ab is allowed to form in the presence of the same dose of antigen(Ag) in the sequential saturation technique than would have been formed in equilibrium analysis (18). The sequential saturation technique will be useful only when the dissociation constant of the Ag · Ab reaction is significantly less than the association constant. The Beckman Twin 10-Test Reagent System for TSH analysis follows the protocol of a sequential addition procedure.

Principles of Assay Design

The essential aim of assay design, is the selection of optimal reagents, assay conditions, and equipment which will provide accurate, reliable laboratory data, in a minimum of time, at minimum cost. The traditional approach to radioimmunoassay design normally involves the preliminary development and isolation of specific antibodies of high affinity, and the synthesis of a labelled antigen of high specific activity. Optimal or suboptimal assay conditions are then developed through mathematical studies and/or laboratory experimentation. When the convenience of a commercial reagent system is employed, the clinical laboratory delegates the responsibility of these preliminary steps to another laboratory, usually unknown. As discussed previously, it is dangerous to accept and implement pre-designed, pre-packaged systems without first testing the reagents, suggested protocol, and assay design under clinical laboratory conditions.

The reagents employed in a radioimmunoassay procedure include an antibody (Ab), the labelled antigen (Ag*), a standard (Ag) or set of standards of known concentration, a reagent or system to separate free and bound antigen and buffer solutions to control pH. It has traditionally been accepted, that to measure low concentrations of a test substance

(Ag), the added amount of labelled antigen (Ag*) should be comparatively small, and the amount of binding reagent (Ab), should be of the same order as that of the Ag (19). The binding energy between antigen and antibody should be such that at the concentration of the reagents employed, the antibody should approach saturation. There is a difference of opinion amongst workers as to the amount of antibody (Ab) that should be employed in a given radioimmunoassay. Berson and Yalow have customarily adjusted the antibody concentration to allow about 50% of the labelled dose of Ag* to be bound in the absence of unlabelled antigen (19). These conditions are applied in a large number of radioimmunoassays. Ekins, however has emphasized that the concentration of reagents, the degree of antibody saturation, and the percent bound in the absence of unlabelled antigen (B_0), depend on which equilibrium technique is being used or whether or not the sequential procedure is employed (19). When only trace concentrations of Ag* are used, the antibody is diluted to bind 33% of the tracer when unlabelled Ag concentration is zero (17). For sequential addition procedures using relatively large amounts of labelled antigen introduced following a preincubation of Ag and Ab, the fraction of total tracer bound may fall to very low levels (less than 10%), in the absence of unlabelled antigen (17,18,19).

Despite these variations in reagent concentration and the percent bound (B_0), there are a number of qualitative conditions required by all techniques. The behaviour of the standard and unknown antigen must be identical in their ability to bind the antibody and compete versus the labelled antigen for the antibody binding sites. However, it is not essential that the label (Ag*), and the unknown or standard (Ag) behave identically, provided the label reacts with identical sites on the antibody, and its distribution between free and bound antigens is a measure

of the concentration of the unlabelled antigen (Ag) in the test system (19,20). Although it is not essential that the labelled antigen be chemically or radioactively pure, impurity can lead to a reduction in sensitivity, precision and accuracy. The specific activity of the labelled compound need not be known with great precision, however it must be sufficiently high to give adequate counts guaranteeing high counting accuracy (20). The antibody (Ab) must fulfill two basic requirements. It should react specifically with the antigen (Ag) and it should be saturable. Lack of specificity may be due to the presence of other compounds which compete for the antibody reaction sites, or "non-specific" elements which effect the energy of reaction between the antigen and antibody. Non-specific interference caused by differing protein concentrations, salts, urea, or changes in pH have been recognized (16).

Accuracy and precision are also dependent on optimal incubation conditions. When an "equilibrium technique" is used, it is important that the incubation temperature and time be such that the system is allowed to reach a state of mass equilibrium. For TSH measurements this has required up to five days (4,9). Increased temperatures and shorter times can be employed, however sensitivity and precision are usually sacrificed. Incubation conditions are also dependant upon the concentration of reactants used. The "sequential protocol" requires that the incubation conditions in step one, allow full binding of the antigen (Ag) and antibody (Ab) to occur. The time employed should allow sufficient formation of the Ag-Ab complex, such that its value becomes experimentally indistinguishable from that reached at perfect equilibrium (17). The incubation time of step two, beginning with the addition of labelled antigen (Ag*) is determined by the time needed to reach sufficient binding Ag* and the Ab sites left unbound after incubation one. Thus

the conditions for the second incubation are determined by the concentration of Ag* and the available Ab following the first incubation step.

The nature of the separation system, can also play a dominant role in determining a procedures accuracy and precision. Ideally the separation method should separate the bound and free fractions totally and instantaneously, halting association and dissociation reactions. The efficiency of the separation should be unaffected by incubation mixture constituents (20,21). Under optimal conditions the "double antibody method" satisfies most of the criteria mentioned, however the influence of various factors must be considered. The precipitating antiserum (second antibody) should be present in concentrations which avoid incomplete precipitation of bound hormone due to either antigen or antibody excess relative to the second antibody reaction (22). Cross reaction of the second antibody with human gamma globulin may also cause incomplete precipitation of bound hormone. The effect of serum proteins, anticoagulants and complement should also be considered. These problems can be mitigated by employing low concentrations of test serum, adequate precipitation times and optimal concentrations of primary and secondary antibodies, checking for anticoagulant effects (21). The conditions of centrifugation for the separation of antibody-bound from free hormone, can also influence assay results. Insufficient washing allowing free labelled hormone to be trapped in the precipitate can introduce important errors if neglected (22).

Finally, the technical procedure employed, including pipetting, manipulation of reaction tubes, and the measurement of radioactivity, are sources of error. Technical errors can be reduced by employing high quality laboratory equipment and pipetting devices, and by giving careful attention to technique. Statistical counting errors are conventionally

reduced by the use of a high specific activity tracer, and/or relatively extended counting times such that 10,000 counts are obtained for each tube (2σ counting error = 2%). It can be concluded that for each radioimmunoassay there will exist optimal concentrations of labelled antigen, antibodies (primary, and secondary) and optimal incubation conditions which maximize the precision, sensitivity, accuracy and specificity of the measurement. These parameters will be evaluated for the Beckman Twin 10-Test Reagent System. The magnitude and variation of the experimental error implicit in the assay procedure will be measured and discussed.

PROCEDURE: BECKMAN TWIN 10-TEST REAGENT SYSTEM

Principle

The Beckman Twin 10-Test Reagent System is designed for the quantitative radioimmunoassay of human thyroid stimulating hormone (HTSH), using the double antibody method (23). The test procedure follows the protocol of a sequential saturation system:

Step I: Unlabelled HTSH and HTSH antibody are incubated together for two hours at 37°C.

Step II: ^{125}I-labelled HTSH is added to the incubation mixture and incubation continues at 37°C for three hours.

Step III: A secondary antibody is added, and the mixture is incubated at 4°C overnight (15-18 hours). HTSH-antibody precipitates, facilitating the separation of bound HTSH from free.

The ^{125}I-labelled HTSH and the unlabelled HTSH (in patients sera or standard preparations) sequentially fill the binding sites of the HTSH antibody. The addition of the secondary antibody precipitates the primary anti-HTSH including the bound HTSH (labelled and unlabelled). The precipitate is then collected by centrifugation, washed, and the radioactivity of the bound ^{125}I-HTSH is counted in a gamma counter. The number of counts in the precipitate, expressed as a percent of total counts of ^{125}I-HTSH added, bear an inverse relationship to the concentration of unlabelled HTSH in each tube. The HTSH concentration in patient and control sera can be determined in micro-International Units per ml. (μI.U./ml) from the standard curve (% bound versus log HTSH). A typical standard curve is shown in Figure 3. The principle of the Beckman system can be represented by the reaction equations presented in Figure 4.

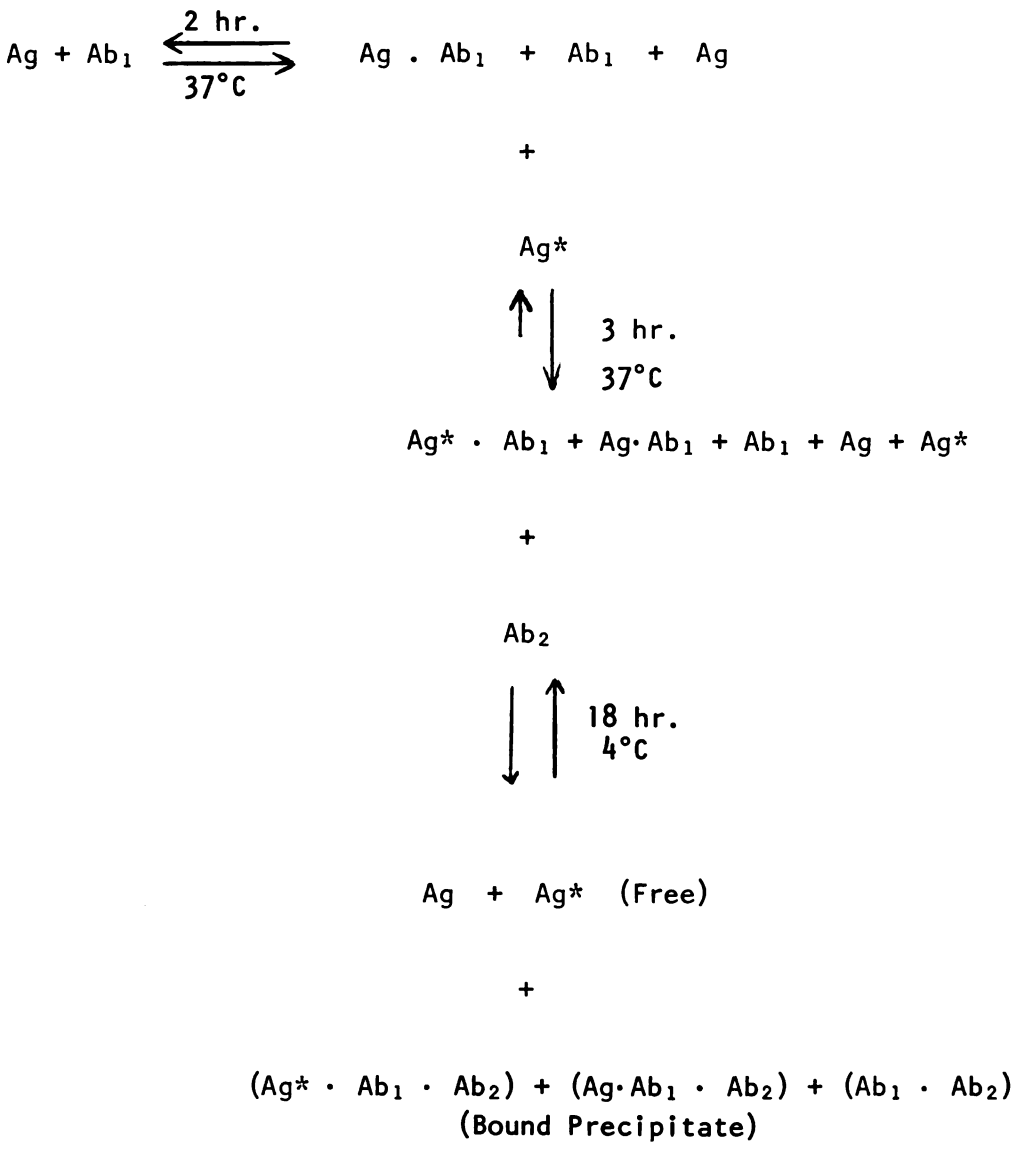


Figure 4: Reaction equations representing the principle employed in the Beckman Twin 10-Test Reagent System for the assay of human thyroid stimulating hormone where:

Ag*	is ^{125}I-labelled HTSH
Ag	is unlabelled HTSH
Ab_1	is HTSH antibody
Ab_2	is precipitating antibody

REAGENTS (23)

1. Beckman Twin 10-Test Reagent System, order no. 566137, Beckman Instruments Inc., Fullerton, California. Each Reagent System contains two vials of each of the following:

(a) ^{125}I-labeled HTSH, Lyophilized.

Each vial contains human thyroid stimulating hormone labeled with less than one microcurie of ^{125}I in rabbit gamma globulin. When reconstituted according to directions and used in accordance with the Beckman instructions, each vial contains "sufficient rabbit gamma globulin to ensure precipitation of the primary antibody by the precipitating antibody." After reconstitution, each vial contains 0.01% sodium azide as preservative. Information does not provide the concentration of HTSH iodinated, or the specific activity of the labelled HTSH.

(b) HTSH Control Serum, Lyophilized.

Each vial contains human serum containing the amount of human thyroid stimulating hormone specified on the label, expressed in International Units per ml. After reconstitution, each vial contains 0.01% sodium azide as preservative.

(c) HTSH Standard, Lyophilized.

Each vial contains 128 micro International Units per ml of human thyroid stimulating hormone in HTSH buffer, pH 7.6. After reconstitution, each vial contains 0.01% sodium azide as preservative.

(d) HTSH Buffer, pH 7.6, Lyophilized.

Each vial contains ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA), fraction V, in phosphate-buffered saline. When reconstituted according to directions, each vial contains; 0.01 molar phosphate, 0.005 molar EDTA, 0.15 molar sodium chloride, 1.0 percent BSA.

(e) Antiserum (rabbit) To HTSH, Lyophilized.

Each vial contains antiserum to human thyroid stimulating hormone produced in rabbits. When reconstituted according to directions and used in accordance with the Beckman instructions, "the amount of antiserum per tube will allow HTSH value from 1.5 µI.U./ml to 128 µIU./ml to be assayed." After reconstitution, each vial contains 0.01% sodium azide as preservative.

(f) Precipitating, Antibody (goat antirabbit gamma globulin), Lyophilized.

Each vial contains serum from goats which have been immunized with rabbit gamma globulin. When reconstituted according to directions and used in accordance with the Beckman instructions, the amount of precipitating antibody per tube will be "sufficient to precipitate the primary antibody present." After reconstitution, each vial contains 0.01% sodium azide as preservative.

2. 0.85% NaCl

EQUIPMENT AND SUPPLIES

1. Oxford Samplers and disposable plastic tips (10, 20, 50, 100, 200, 300, 500, 1000 µl). Oxford Laboratories, San Mateo, California.
2. Test Tubes, plastic, 12 x 75 mm. Beckton Dickinson #2052, Beckton Dickinson and Company, Oxnard, California.
3. Refrigerated Centrifuge, Sorvall RC-3 General Purpose Automatic; Ivan Sorvall Inc., Santa Monica, California.
4. Automatic Gamma Counting System, Nuclear-Chicago 1185 Series.
5. 38°C Waterbath.
6. Vortex-type mixer.
7. Refrigerator-freezer.

TEST PROCEDURE (23)

1. Approximately thirty minutes before the assay is to be run, remove one vial of each reagent system component from the refrigerator and reconstitute with sterile distilled water as follows:
   (a) 5 ml ^{125}I-labeled HTSH
   (b) 1.00 ml HTSH control serum
   (c) 2.00 ml HTSH standard
   (d) 20.0 ml HTSH buffer
   (e) 5.0 ml Antiserum to HTSH
   (f) 5.0 ml Precipitating antibody
2. Label reconstituted HTSH standard from Step 1(c) immediately preceding, as A. Label seven (7) 12 x 75 mm test tubes B through H. Serially dilute the standard with the reconstituted buffer from Step 1(d) immediately preceding, as follows:

STANDARD	SOLUTION	CONCENTRATION µI.U./ml
A	Reconstituted HTSH standard	128
B	1.0 ml of A + 1.0 ml of buffer	64
C	1.0 ml of B + 1.0 ml of buffer	32
D	1.0 ml of C + 1.0 ml of buffer	16
E	1.0 ml of D + 1.0 ml of buffer	8
F	1.0 ml of E + 1.0 ml of buffer	4
G	1.0 ml of F + 1.0 ml of buffer	2
H	1.0 ml of G + 1.0 ml of buffer	1

3. Label 24 vials, in duplicate, as follows: T.C., blank, B_0, A through H, and CS.

   where: T.C. is total count,
   blank is counts bound in absence of primary antibody,
   B_0 is counts bound in absence of unlabelled HTSH,
   A through H are the standards, and
   CS is control serum.

4. Label 20 vials in duplicate, 1 through 10, for patient samples to be tested.

5. Add 300 µl of buffer to the blank vials.

6. Add 200 µl of buffer to the B_0 vials.

7. Add 200 µl of standards A through H to the appropriate vials.

8. Add 200 µl control serum to CS vials.

9. Add 200 µl of each patient's serum to the appropriate tubes (For patients with primary hypothyroidism, it may be necessary to dilute the patient sample 1/10 in buffer before assay.)

10. Add 100 µl of dilute HTSH antiserum to all vials except T.C. and blank.
11. Cap all vials except T.C., and mix thoroughly by gentle swirling or gentle vortexing.
12. Incubate for two (2) hours at 37°C (except T.C. vials).
13. Add 100 µl of ^{125}I-HTSH to all vials. Cap T.C. vials and set aside. Cap other vials and mix thoroughly by gentle swirling or gentle vortexing.
14. Incubate for three (3) hours at 37°C.
15. Add 100 µl of precipitating antiserum to all vials (except T.C.). Cap vials and mix thoroughly by gentle vortexing.
16. Incubate for 15 to 18 hours (overnight) at 4°C.
17. Add 1 ml of cold (4°C) 0.85% saline to each vial (except T.C.). Cap vials and mix thoroughly by gentle swirling or gentle vortexing.
18. Immediately centrifuge all vials (except T.C.) for 30 minutes at 3000 x g, or 15 minutes at 6000 x g.
19. Decant each vial carefully and discard supernatant (except T.C.)
20. Add 2 ml of cold (4°C) 0.85% saline to the precipitate in each vial (except T.C.). Then cap vials and mix by gentle swirling or gentle vortexing.
21. Immediately centrifuge all vials (except T.C.) for 30 minutes at 3000 x g or 15 minutes at 6000 x g.
22. Carefully decant each vial and discard supernatant (except T.C.).
23. Count *all* vials, including T.C., for a length of time such that at least 10,000 counts (2σ counting error = 2%) are collected for each vial. This will be somewhere between 1 and 10 minutes. For convenience, counting can be delayed after Step 20, however, it is

recommended that the counting be done as soon as practical after completion of the test.

The protocol is summarized in Table 2.

CALCULATIONS

The counts obtained in Step 21 for the standards and patient samples, are converted to percent of counts bound to antibody, %B, by the following formula:

$$\%B = \frac{\text{Counts (or cpm) for standard, control serum, or patient's sample} - \text{blank}}{\text{T.C.} - \text{blank}} \times 100$$

For purposes of this calculation, the value of T.C. to be used in the above equation is the average of the duplicate total counts, and the value for the blank is the average of the duplicate blank counts.

The values of %B are calculated for B_0, the standards, the control serum, and the patient samples. A separate value for each duplicate should be obtained.

A standard curve is obtained by plotting the values of the %B for the standards versus the log of the concentration of the HTSH in the standards in μI.U./ml. The curve is plotted on three (3) cycle semilog graph paper, with the %B as the linear ordinate and the quantity of HTSH as the log abscissa. The value for %B for B_0 corresponds to zero HTSH. The duplicate values for %B are plotted separately (at the same value of HTSH) and a "best fit" curve is drawn through all the points.

The values of the HTSH level for the patient's samples and the control serum are obtained by averaging the duplicate values of the %B and reading the value of HTSH in μI.U./ml from the standard curve. If the difference in the values of %B for any two duplicates is greater than 30% of the average value of %B for the same duplicates, the value of HTSH level for

that sample should not be used. The test for that particular sample should be repeated. The value obtained for the control serum must be within the limits stated on the control serum vial.

SAMPLE (in Duplicate)	BUFFER (µl)	STANDARD OR SAMPLE (µl)	DILUTE ANTI-HTSH (µl)	^{125}I-HTSH (µl)	PRECIPITATING ANTISERA (µl)
T.C.	0	0	0	100	0
Blank	300	0	0	100	100
B_0	200	0	100	100	100
A(128 µI.U./ml)	0	200-A	100	100	100
B(64 µI.U./ml)	0	200-B	100	100	100
C(32 µI.U./ml)	0	200-C	100	100	100
D(16 µI.U./ml)	0	200-D	100	100	100
E(8 µI.U./ml)	0	200-E	100	100	100
F(4 µI.U./ml)	0	200-F	100	100	100
G(2 µI.U./ml)	0	200-G	100	100	100
H(1 µI.U./ml)	0	200-H	100	100	100
Control Serum	0	200-CS	100	100	100
Patient Sample 1	0	200-Sample	100	100	100
Patient Sample 2	0	200-Sample	100	100	100
etc.	0	200-Sample	100	100	100
	0	200-Sample	100	100	100

$$\%B = \frac{\text{Counts (or cpm) for standard, control serum, or patient's sample} - \text{blank}}{\text{T.C.} - \text{blank}} \times 100$$

Table 2: PROTOCOL BECKMAN - TWIN 10-TEST REAGENT SYSTEM (23)

BECKMAN HTSH SYSTEM: EXPERIMENTS AND RESULTS

A. Evaluation of Assay Protocol and Design

Comparison of Sequential Saturation and Equilibrium Analysis

The Beckman HTSH procedure follows the protocol of a sequential saturation system. Dose-response curves obtained using the sequential saturation technique were compared to curves obtained when the Beckman reagents were used under "equilibrium conditions." The HTSH standards and ^{125}I-labelled HTSH were incubated simultaneously with the anti-HTSH. Following an overnight incubation at 37°C, the precipitating antibody was added and the mixture incubated eighteen hours at 4°C. The bound HTSH was then separated from the free HTSH and the radioactivity of each measured. At zero dose HTSH, the percent bound was identical (B_0=18.5%) for both techniques. At low concentrations, each finite dose of HTSH resulted in a greater decrease in % Bound when the sequential saturation technique was employed. A steeper dose response curve resulted and greater sensitivity was realized with the Beckman sequential saturation protocol (See Figure 5).

Incubation of Unlabelled HTSH and Anti-HTSH Antibody

The first step of the Beckman procedure involves a two hour incubation of unlabelled HTSH and anti-HTSH antibody at 37°C. Decreasing the incubation time by one hour resulted in reduced precision and decreased sensitivity. Increasing the incubation to 2 1/2 - 3 hours had no effect. The binding of unlabelled HTSH and anti-HTSH antibody at 37°C reached completion within two hours. When the incubation temperature was reduced to 25°C a decrease in sensitivity was observed.

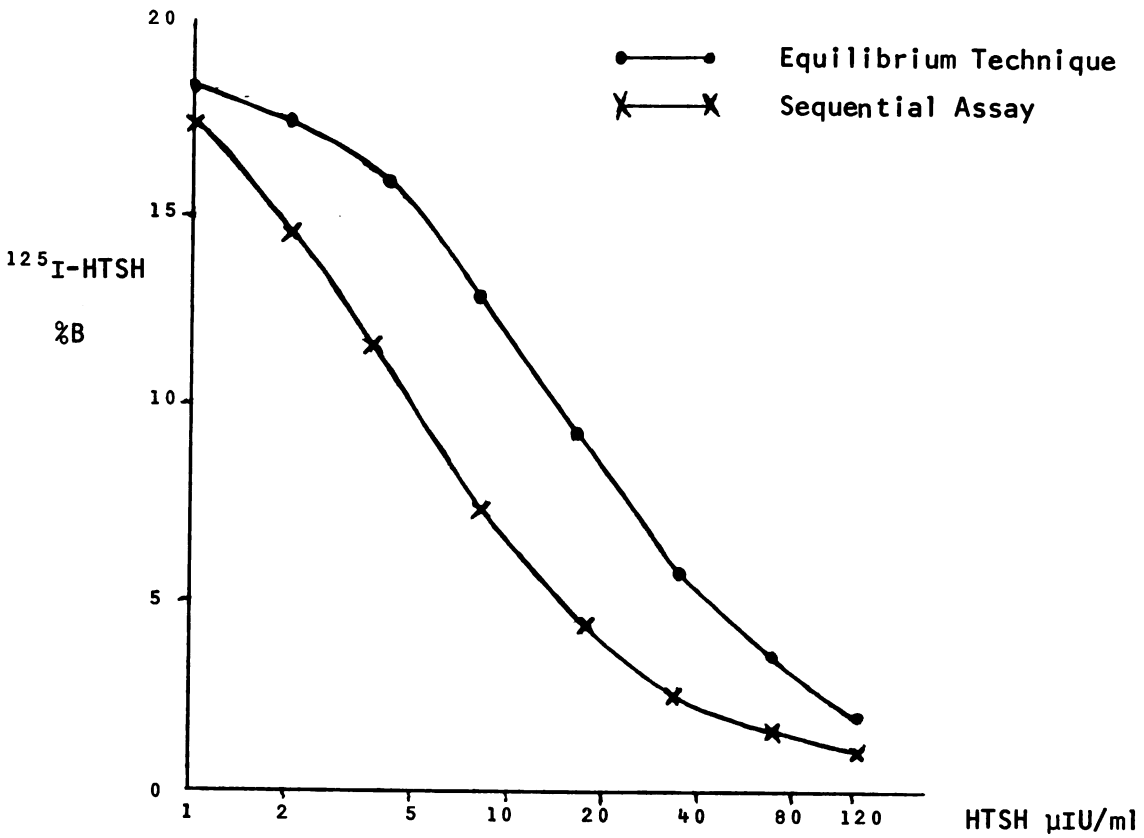


Figure 5: HTSH Dose-response curves obtained by equilibrium and sequential saturation techniques.

The Sequential Addition of ^{125}I-Labelled HTSH

The incubation conditions for step two of the HTSH procedure were studied. The association of the labelled antigen with its antibody was followed by mixing ^{125}I-HTSH with anti-HTSH antibody and interrupting the binding process at increasing time intervals with the addition of the precipitating antibody. Precipitation of the bound HTSH was allowed to proceed overnight at 4°C. Bound HTSH was separated by centrifugation, washed, and the radioactivity counted. The resulting binding curve is shown in Figure 6. The binding reaction is essentially complete at three hours. When the precipitating antibody was added at zero time, immediately after the mixing of ^{125}I-labelled HTSH and HTSH antibody, the association reaction continued, reaching 40% B/B_0. Thus it is demonstrated that binding continues after the addition of the precipitating antibody.

To gain insight into the rate of displacement of unlabelled HTSH during the second incubation, the method of Farr(18) was used. Aliquots of antibody and ^{125}I-HTSH were mixed and allowed to come to equilibrium. An excess of unlabelled HTSH was then added. After increasing times of incubation the precipitating antibody was added and the bound HTSH precipitated. Following overnight precipitation the radioactivity of the bound HTSH was determined and the dissociation curve plotted (Figure 7). Under the assay conditions described, approximately 18% of the ^{125}I-HTSH originally bound to the antibody was found to dissociate. Figure 7 would suggest timing for the three hour incubation is not critical. To avoid variation in the extent of redissociation from one test tube to the other the timing of the second incubation was carefully controlled for all test tubes incubated.

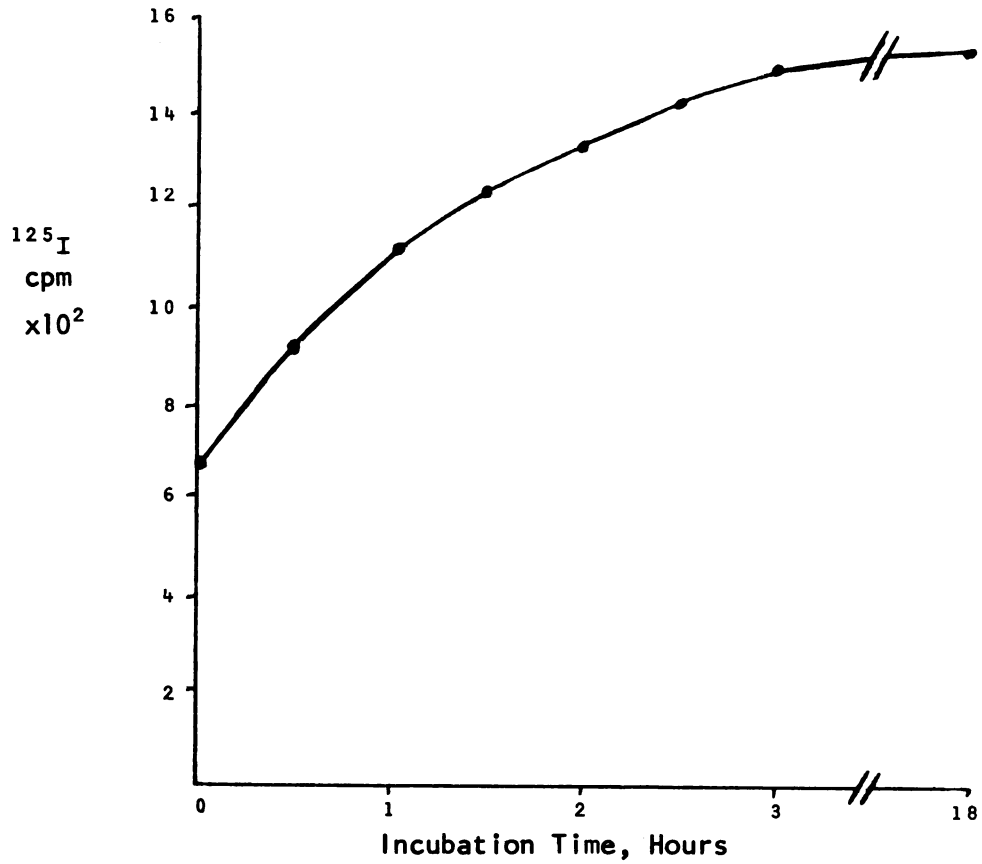


Figure 6: Association Reaction of ^{125}I-HTSH With Anti-HTSH Antibody at 37°C.

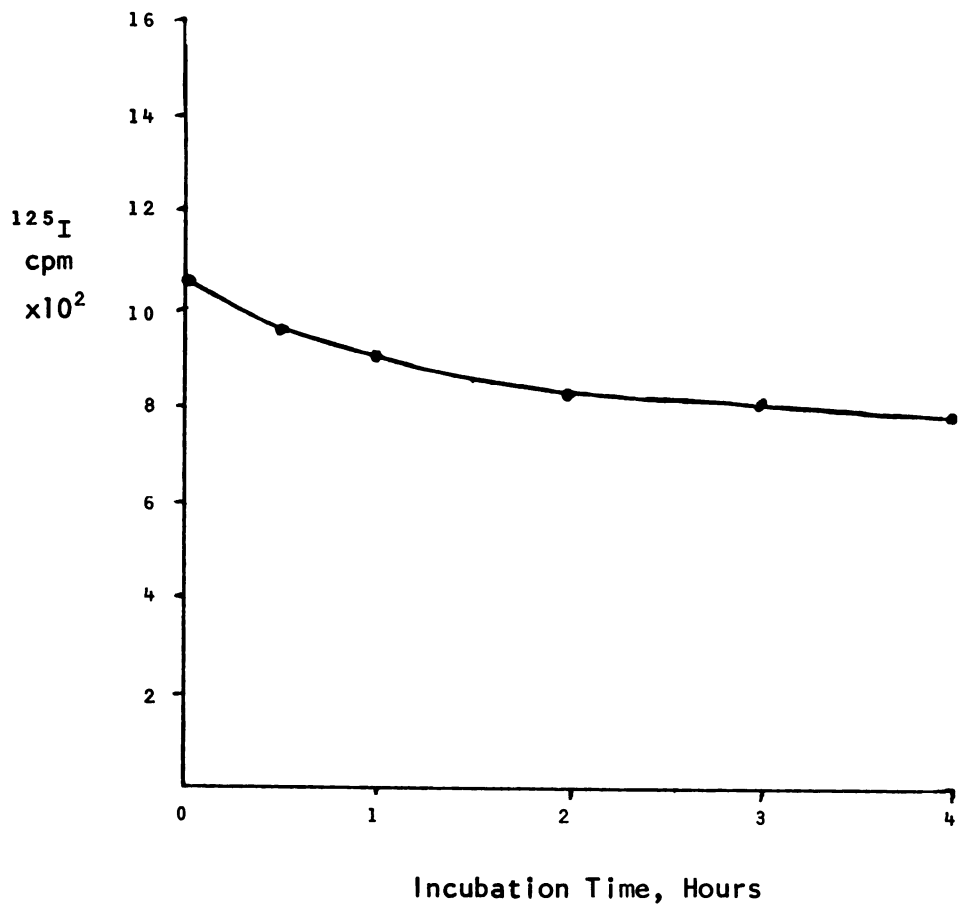


Figure 7: Dissociation Reaction of ^{125}I-HTSH From Its Complex With Antibody

Separation of Bound and Free HTSH

Following the incubation of ^{125}I-labelled HTSH, unlabelled HTSH and the anti-HTSH antibody, the precipitating antibody is added and the mixture incubated overnight (15-18 hours) at 4°C. The precipitated HTSH antibody is collected by centrifugation, washed with cold saline and the radioactivity of the bound ^{125}I-HTSH determined. Zero dose standards were assayed and the precipitation reaction terminated at hourly intervals. The percent ^{125}I-HTSH bound was determined. As shown in Table 3, B/B_0 increased from about 65% after one hour of incubation to 84% at four hours. Since the HTSH protocol does not allow addition of the precipitating antibody until some 5-6 hours after beginning the procedure, it was more convenient to allow the precipitation reaction to continue overnight than to terminate the precipitating reaction at 6-8 hours. It was found that timing was not critical for the overnight incubation, precipitation being complete at 15 hours. The relative volumes of first and second antibody combined in the reaction tubes was found to be critical. For one set of reagents tested, only 40% of the labelled hormone was precipitated when the volume of anti-HTSH antibody was doubled (ie. 200 µl). Other reagent sets tested resulted in precipitation of 89-92% of the labelled antigen when the first antibody was doubled. Varying the volume of the precipitating antibody also affected the precipitation of bound-HTSH. This data is presented in Table 4.

The conditions of centrifugation and washing for the separation of antibody-bound hormone and free hormone were evaluated. Cold saline was added to the reaction tubes following overnight precipitation. The tubes were vortexed and then centrifuged 45 minutes at 3000 rpm using a refrigerated centrifuge. Figure 8 demonstrates that one wash is insufficient

Incubation Time Hours	Precipitated ^{125}I-counts	^{125}I-HTSH % Bound	^{125}I-HTSH % B/B_0
1	9317	12.2	65
2	10702	14.2	76
3	11428	15.2	81
4	11723	15.7	84
15	13850	18.7	100
16	13826	18.7	100
18	13780	18.6	99.5

Table 3: Effect of Time on the Precipitation of ^{125}I-labelled HTSH.

Volume, µl Ab_2	Precipitated ^{125}I-counts	^{125}I-HTSH % Bound	^{125}I-HTSH % B/B_0
80	22797	19.2	54
100	40817	35.8	100
120	39870	34.3	96
140	39700	34.1	95
160	38763	33.3	93
180	37106	31.8	89
200	37150	32.0	89

Table 4: Effect of Precipitating Antibody (Ab_2) volume on the precipitation of ^{125}I-Labelled HTSH. The different volumes of Ab_2 were added to reaction tubes containing 100 µl anti-HTSH, 200 µl buffer, and 100 µl ^{125}I-HTSH.

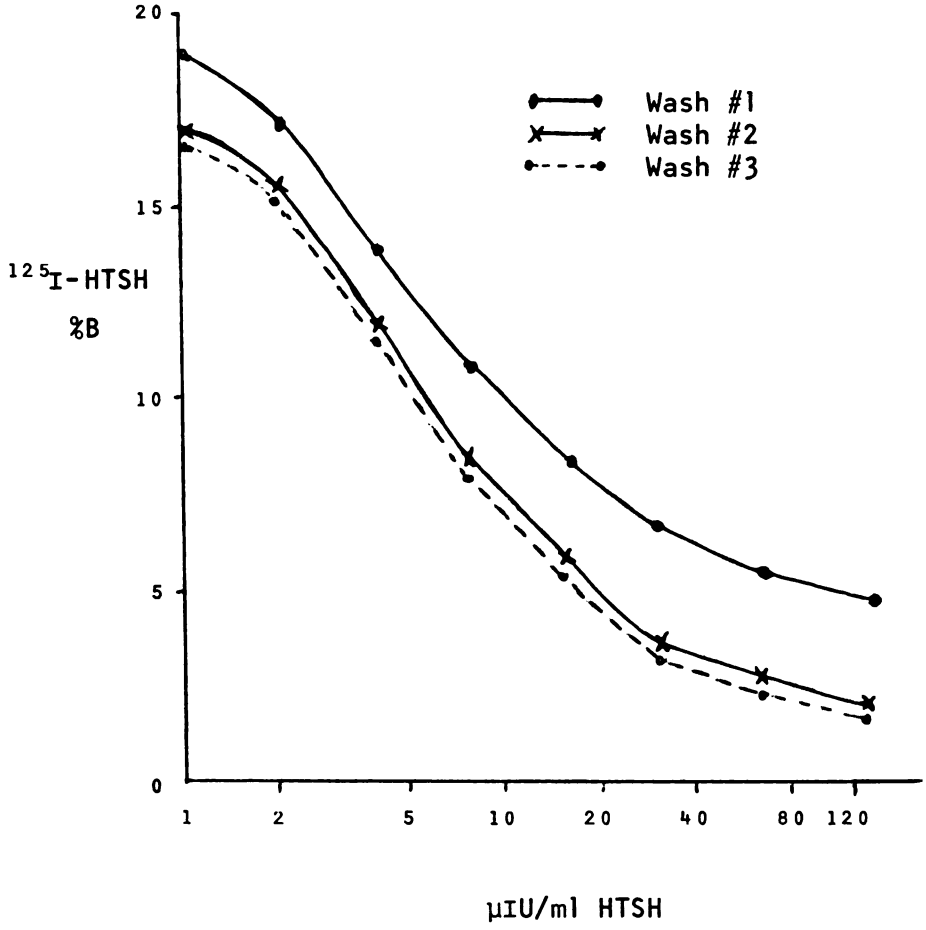


Figure 8: Influence of Centrifugation-Wash Conditions on the HTSH Dose-Response Curve.

resulting in the displacement of the standard curve. The centrifugation-wash procedure was repeated a second time. The amount of radioactivity bound to the surface of the tubes and trapped in the precipitate was significantly reduced. A third wash was less effective and did not displace the standard curve appreciably. Considering the time and labour involved, it was not found worthwhile to wash the precipitate a third time. The non-specific radioactivity trapped in the precipitate following two washes was assessed in control tubes containing no first antibody. It was found to be less than 1% of the total radioactivity added. Using two washes it was found that the precipitate collected was easy to handle, while low blanks and good precision were achieved.

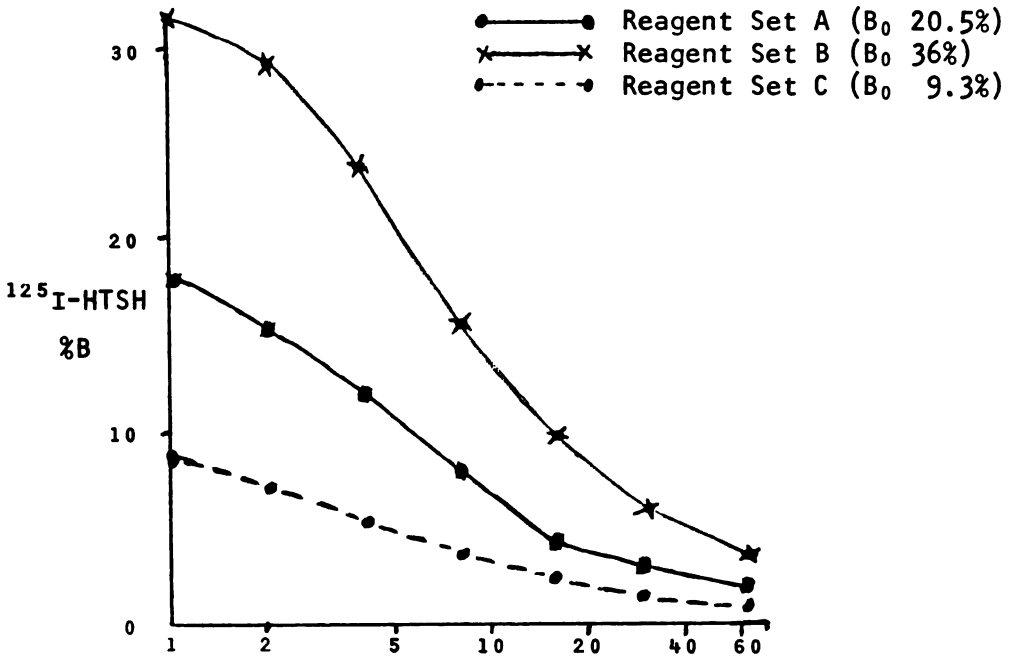
Calculations and Data Handling

As previously discussed, there are several ways of expressing the dose response phenomenon. For the Beckman double antibody procedure it is convenient to calculate percent bound (%B). When percent bound is plotted on the linear scale versus dose HTSH on the log scale, a sigmoidal curve results. Rodbard et.al. (24,25,26) have used the logit tranformation to linearize sigmoidal dose response curves plotting logit (B/B_0) versus log dose. The logit function was applied to the HTSH data, however the transformation did not provide satisfactory linearization. Patient results calculated using the logit plot differed significantly from those calculated using the sigmoidal curve. For the HTSH procedure it was more valid to include an increased number of standards in the assay, connecting each pair of consecutive standard points by a straight line. Thus it was not assumed that an equation could be fitted to the standard curve.

HTSH Standard Curve

Standard curve parameters can be a great value in the establishment of an assay (24,27). Figure 9 compares dose-response curves obtained using reagent systems having different lot numbers. Despite the difference in percent binding, when these same curves are plotted B/B_0 versus log HTSH it is seen their general shape, slope, and sensitivity are similar. Control sera at low, medium and high levels of HTSH compared favourably when assayed by each different reagent system. The curve in Figure 10 represents the average of five dose-response curves obtained using the same set of reagents. The standard deviation of each point is given. The data on which Figure 10 is based is presented in Table 5.

Standard curves for the HTSH assay were prepared in different protein solutions. Different amounts of bovine serum albumin (BSA), bovine serum globulin (BSγG), canine serum and calf serum were added to the standard samples. Figure 11 demonstrates the influence of serum proteins on the standard curve. Dose-response curves prepared in phosphate buffer containing 1% and 5% BSA were identical to the curves obtained using standards diluted in the Beckman buffer. Standards analyzed in the presence of calf serum, canine serum, or 5% bovine serum gamma globulin (BSγG) were displaced to the left of the "Beckman curve." Accordingly it is clear that when an unknown serum was read from the Beckman standard curve, a higher value was obtained than that when the curves containing calf serum, dog serum or 5% BSγG were used. Similar findings have been reported by Sluiter (9), Patel (28), and Franchimont (11).



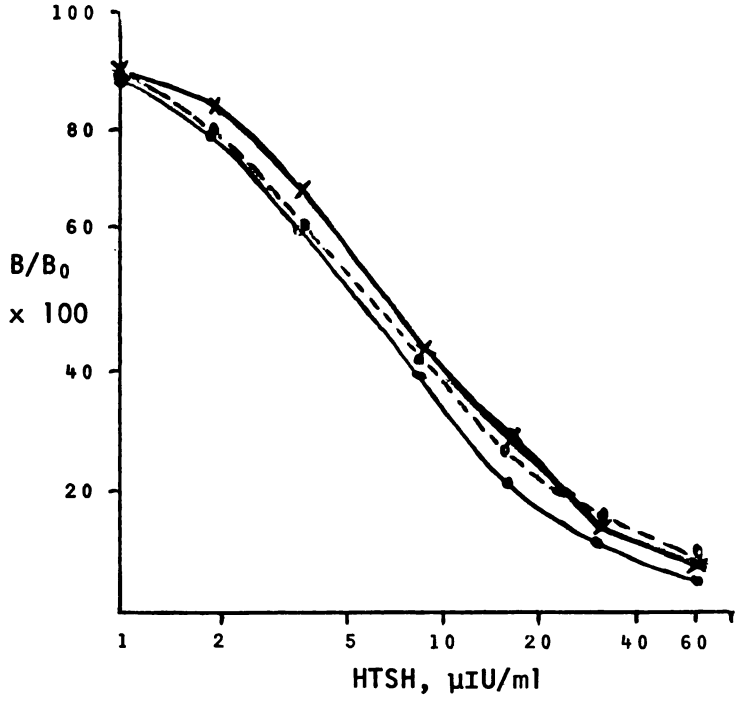


Figure 9: Comparison of dose-response curves obtained using different reagent sets.
Upper Curve: plotting %B versus log HTSH
Lower Curve: plotting % B/B_0 versus log HTSH

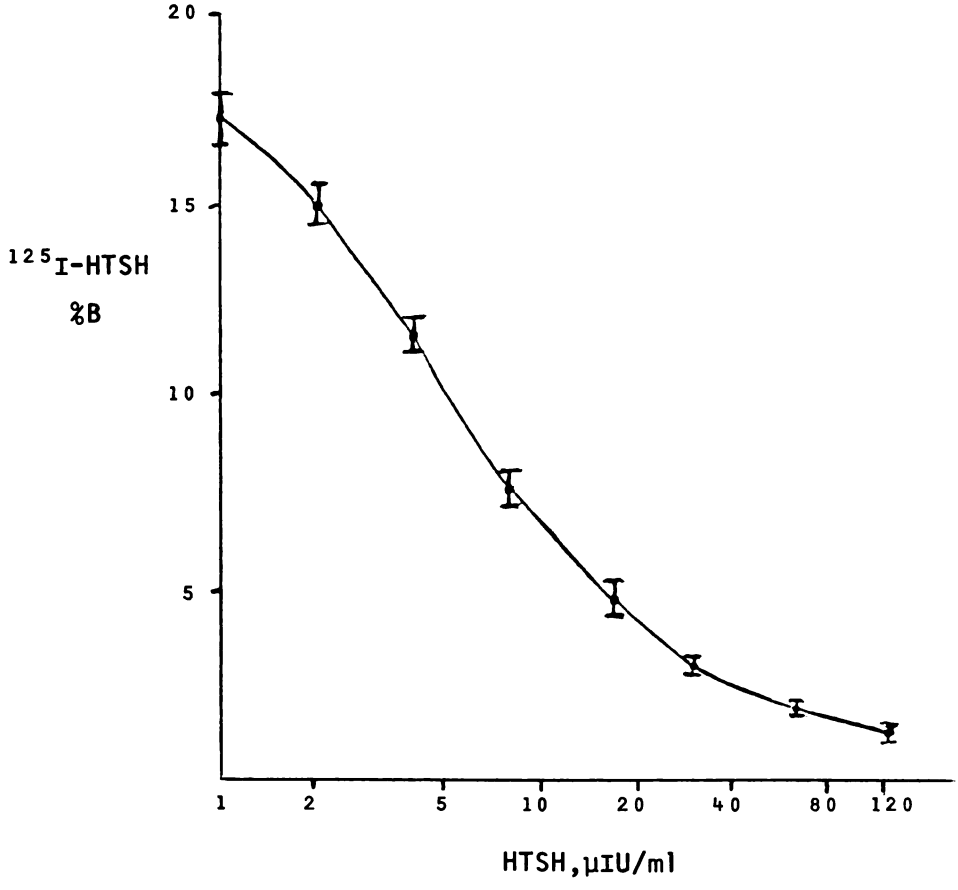


Figure 10: HTSH Standard Curve. Each point denotes the mean of ten determinations. The vertical bars represent ± S.D.

HTSH, μIU/ml	Mean % B	Number of Determinations	Standard Deviation (±SD)	Standard Error of Mean (SEM)
128	1.3	10	0.2	0.06
64	1.9	10	0.12	0.04
32	3.1	10	0.2	0.06
16	4.8	10	0.4	0.13
8	7.7	10	0.4	0.13
4	11.6	10	0.5	0.16
2	15.1	10	0.6	0.19
1	17.3	10	0.7	0.22

Table 5: Values forming the basis of the standard curve given in Figure 10.

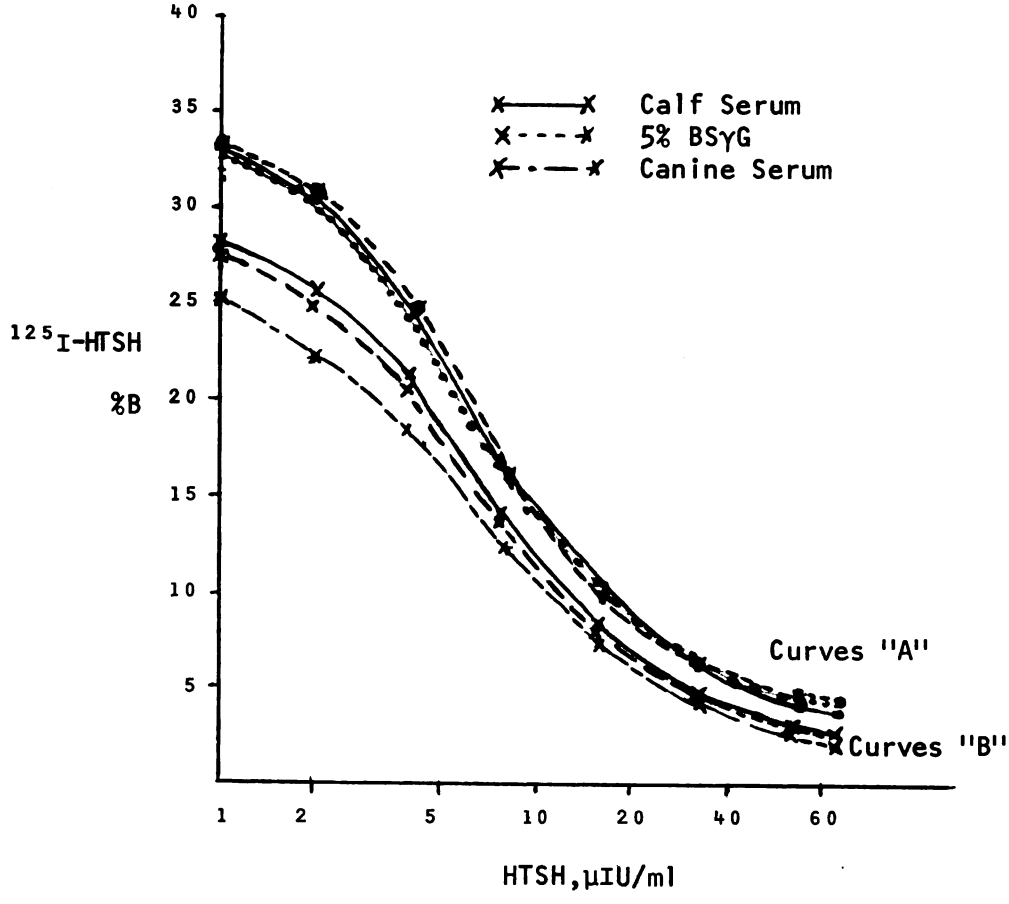


Figure 11: The influence of serum proteins, on the HTSH dose-response curve. Curves "A" includes the dose-response curves containing HTSH Buffer, 5% BSA, and 1% BSA. Curves "B" contain calf serum, canine serum and 5% BSγG.

Micro Adaptation of the Beckman Protocol

Employing the Beckman protocol, each half of the Twin-10 Test Reagent System package contained sufficient reagents to assay the necessary standards and ten patient samples in duplicate. Combining the reagents in the twin-package allowed for the duplicate assay of up to twenty-five patient sera and an eight-point standard curve. It was found that the reagent volumes could be reduced to one-half that of the suggested protocol without altering the characteristics of the standard curve. The results of patient sera assayed by both the macro and micro techniques were identical. The counting time was increased allowing the collection of 10,000 counts for each tube. The volume of saline used in the wash-centrifugation procedure was also reduced by one-half. The modified protocol is presented in Table 2A. Using the micro adaptation, thirty-five patient sera could be assayed in duplicate using one-half of the Twin-10 Test Reagent System. Combining the twin packages, sufficient reagents would be present to assay a single eight-point standard curve and approximately eighty patient sera. The "micro-assay" was adopted for use in the laboratory and is characterized in the reliability studies which follow.

SAMPLE (in Duplicate)	BUFFER (µl)	STANDARD OR SAMPLE (µl)	DILUTE ANTI-HTSH (µl)	^{125}I-HTSH (µl)	PRECIPITATING ANTISERA (µl)
T.C.	0	0	0	50	0
Blank	150	0	0	50	50
B_0	100	0	50	50	50
A(128 µI.U./ml)	0	100-A	50	50	50
B(64 µI.U./ml)	0	100-B	50	50	50
C(32 µI.U./ml)	0	100-C	50	50	50
D(16 µI.U./ml)	0	100-D	50	50	50
E(8 µI.U./ml)	0	100-E	50	50	50
F(4 µI.U./ml)	0	100-F	50	50	50
G(2 µI.U./ml)	0	100-G	50	50	50
H(1 µI.U./ml)	0	100-H	50	50	50
Control Serum	0	100-CS	50	50	50
Patient Sample 1	0	100-Sample	50	50	50
Patient Sample 2	0	100-Sample	50	50	50
etc.	0	100-Sample	50	50	50
	0	100-Sample	50	50	50

$$\%B = \frac{\text{Counts (or cpm) for standard, control serum, or patient's sample} - \text{blank}}{\text{T.C.} - \text{blank}} \times 100$$

Table 2A: Micro Adaptation of the Beckman Protocol

B. Performance Characteristics: Micro Adaptation of the Beckman Protocol

Specificity

Specificity has been defined as "the extent of freedom from interferences by substances other than the one intended to be measured" (29). It has been reported that in almost all antisera raised against HTSH, part of the antibody population shows crossreactivity against human luteinizing hormone (HLH), human follicle stimulating hormone (HFSH) and human chorionic gonadotropin (HCG). These hormones are known to have peptide chains in common (4). Crossreaction studies using purified HCG were performed. Figure 12 compares the dose-response curves obtained using HCG in buffer versus those obtained using the HTSH standard. HCG concentration up to 100 I.U. did not interfere. Crossreactivity was further tested by adding purified preparations of HCG to patient sera. No interference was detected when serum HTSH measurements were performed on sera containing up to 66 I.U. HCG.

The behaviour of the HTSH standard and HTSH in patient serum was assessed by serially diluting patient sera and comparing the dose-response curve obtained with the HTSH standard curve (Figure 13). An alternative test of identity suggested by Ekins (19) was also performed. The potency of the HTSH standard and HTSH in patient sera was compared in assays containing different concentrations of binding reagent. The antibody dilution curves presented in Figure 14 demonstrate parallelism. Tests of parallelism by themselves cannot be interpreted as conclusive evidence of specificity. The effect of protein on the standard curve has already been cited. Other non-specific interfering substances were tested. Results from plasma samples collected using E.D.T.A., and heparin were compared to serum results. There were no significant differences.

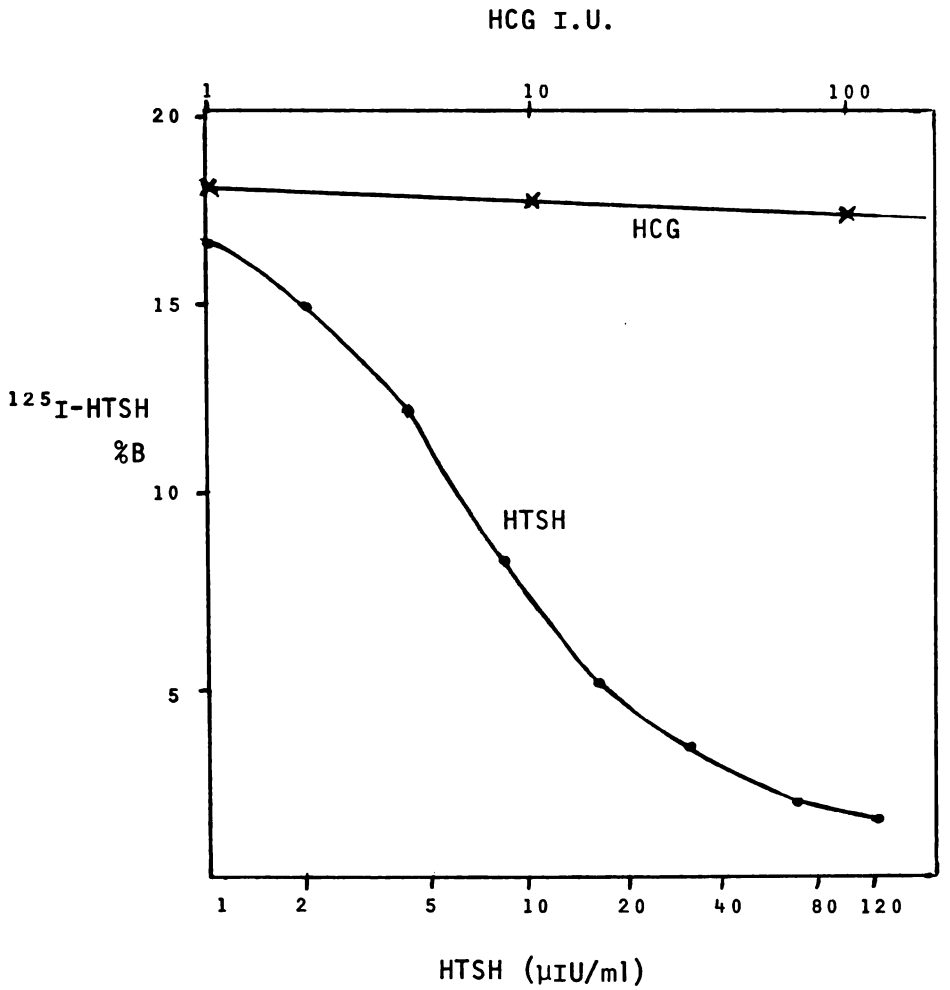


Figure 12: Comparison of dose-response curves obtained for human thyrotropin (HTSH) and purified human chorionic gonadotropin (HCG) using the Beckman HTSH System.

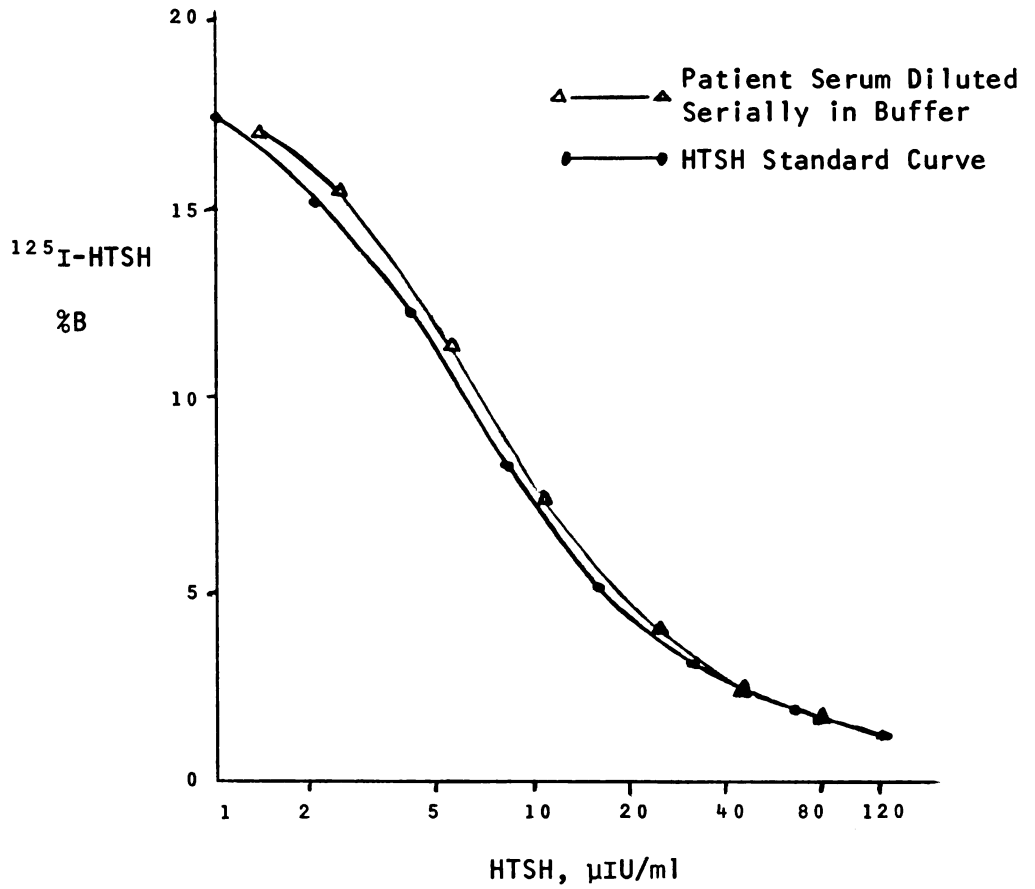


Figure 13: Comparison of the HTSH dose response curve and a patient serum diluted serially.

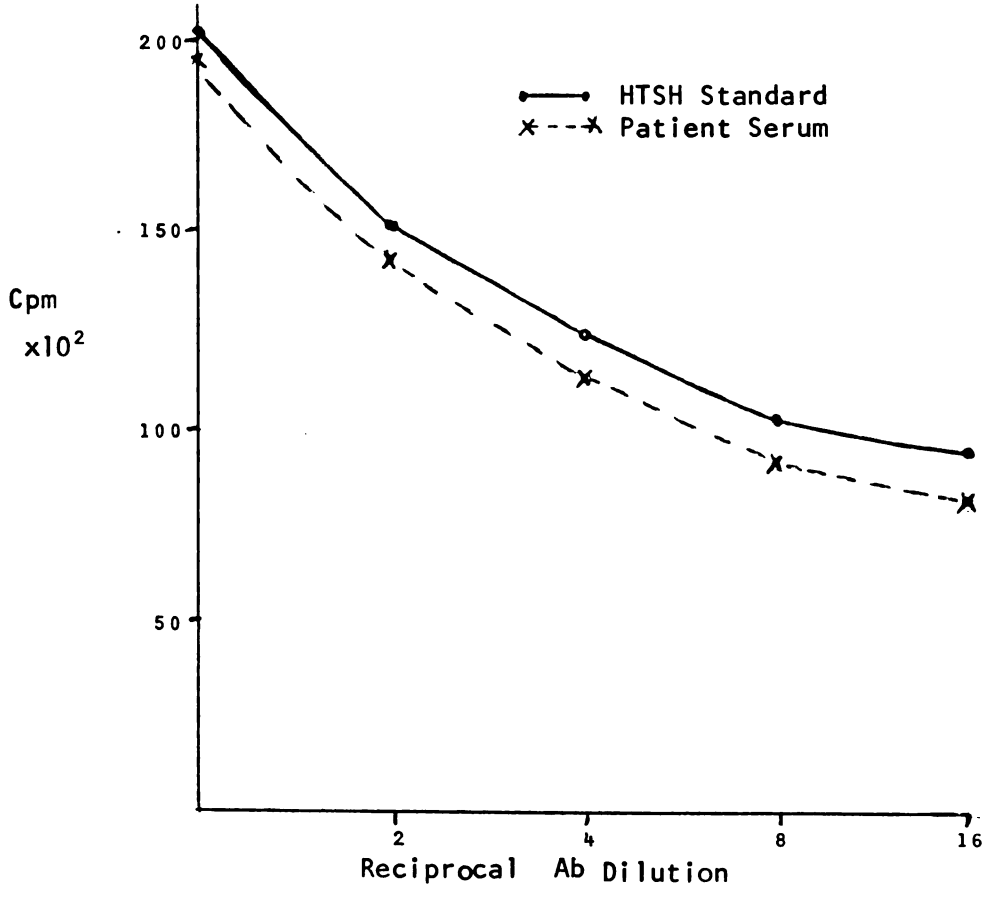


Figure 14: Test of identity. Fixed amounts of unlabelled hormone (patient or standard), and labelled hormone were added to different concentrations of antibody.

Similarily it was demonstrated that urea nitrogen levels up to 60 mg%, and hemolysis visible to the eye did not interfere. Further studies supporting HTSH specificity will be offered. HTSH concentrations in sera drawn from euthyroid, hypothyroid and hyperthyroid patients will be seen to correlate well with "expected" results. The response to administered TSH (200 µg. I.V.) also followed predicted tolerance levels.

Sensitivity

It was found that 1.5 µIU/ml could be assayed with confidence as claimed by Beckman (23). The 1 µIU/ml HTSH standard gave a response significantly different from zero dose HTSH. Table 6 compares percent B/B_0 over the range of standards employed. The average B/B_0 for 1 µIU/ml and 2 µIU/ml HTSH were 86.9% and 75.9% respectively. It was found that samples with HTSH levels over 64 µIU/ml, could not be determined with confidence from the standard curve. The slope of the curve between 64-128 µIU/ml is seen to be relatively flat. Samples over 64 µIU/ml were diluted in buffer and repeated. The precision achieved for each mass point on the dose-response curve was presented in Table 5.

Reproducibility

Within-assay and between-assay variation was measured at critical HTSH dose levels using the statistical assay procedure recommended by Rodbard (26). Samples were analyzed in duplicate in each of several assays. The raw data, intra-assay and inter-assay variability is presented in Tables 7-10. Control sera analyzed at the beginning and end of HTSH assay sets were identical.

HTSH µIU/ml	Precipitated ^{125}I - cpm	%B	%B/B_0
128	207	1.2	6.0
64	261	1.8	9.0
32	361	3.1	15.6
16	535	5.3	26.6
8	735	7.8	39.2
4	1048	11.8	59.3
2	1312	15.1	75.9
1	1486	17.3	86.9
0	1687	19.9	100
	Total Counts	8034 cpm	
	Blank Counts	115 cpm	

Table 6: HTSH Assay Data

Assay	HTSH RESULTS IN µIU/ml Duplicates X_1	X_2	Mean, $\bar{X}$	ΔX	Standard Deviation
1	70	70	70	0	0
2	64	64	64	0	0
3	64	66	65	-2	1.42
4	64	66	65	-2	1.42
5	64	64	64	0	0
6	64	63	63.5	+1	0.71
7	70	72	71	-2	1.42
8	66	65	65.5	+1	0.71
9	64	66	65	-2	1.42

	Standard Deviation	Coefficient of Variation
Within assays		
For single tube	1.0	1.5%
For means of duplicates	0.7	1.1%
Between assays		
For means of duplicates	2.5	3.8%

Table 7: Intra-assay and Inter-assay Variance
Control Serum, $\bar{X}$ = 65.9 µIU/ml

Assay	HTSH RESULTS IN µIU/ml Duplicates X_1	X_2	Mean, $\bar{X}$	ΔX	Standard Deviation
1	5.4	6.0	5.7	-0.6	0.43
2	5.2	5.2	5.2	0	0
3	5.5	5.6	5.55	-0.1	0.07
4	5.2	4.9	5.05	-0.3	0.21
5	5.4	5.0	5.2	+0.4	0.28
6	5.2	5.2	5.2	0	0
7	6.0	5.6	5.8	+0.4	0.28
8	5.8	5.6	5.7	+0.2	0.14
9	6.2	5.8	6.0	+0.4	0.28
10	4.8	4.5	4.65	+0.3	0.21

	Standard Deviation	Coefficient of Variation
Within assays		
For single tube	0.23	4.3%
For means of duplicates	0.16	3.0%
Between assays		
For means of duplicates	0.41	7.6%

Table 8; Intra-assay and Inter-assay Variance, Patient Serum A, $\bar{X}$ = 5.4 µIU/ml

HTSH RESULTS IN µIU/ml

Assay	Duplicates X_1	Duplicates X_2	Mean, $\bar{X}$	ΔX	Standard Deviation
1	2.8	2.8	2.8	0	0
2	2.7	2.9	2.8	-0.2	0.14
3	3.2	3.6	3.4	-0.4	0.28
4	3.5	3.6	3.55	-0.1	0.07
5	3.9	3.7	3.8	+0.2	0.14
6	3.8	3.6	3.7	+0.2	0.14
7	3.4	3 5	3.45	-0.1	0.07
8	3.4	3.5	3.45	-0.1	0.07
9	4.4	3.9	4.15	+0.5	0.35
10	3.9	3.6	3.75	+0.3	0.21

	Standard Deviation	Coefficient of Viariation
Within Assays		
For single tube	0.18	5.2%
For means of duplicates	0.13	3.7
Between assays		
For means of duplicates	0.4	11.5%

Table 9: Intra-assay and Inter-assay Variance

Patient Serum B, $\bar{X}$ = 3.5 µIU/ml

Assay	HTSH RESULTS IN µIU/ml Duplicates X_1	X_2	Mean, $\bar{X}$	ΔX	Standard Deviation
1	19	20	19.5	-1	0.7
2	20	18	19	+2	1.4
3	22	22	22	0	0
4	21	26	23.5	-5	3.5
5	22	22	22	0	0
6	22	24	23	-2	1.4

	Standard Deviation	Coefficient of Variation
Within assays		
For single tubes	0.57	2.7%
For means of duplicates	0.40	1.9%
Between assays		
For means of duplicates	1.8	8.4%

Table 10: Intra-assay and Interassay Variance
Patient Serum C, $\bar{X}$ = 21.5 µIU/ml

Accuracy

The accuracy of the assay was tested by the recovery of unlabelled HTSH added to buffer and to serum. Recoveries in buffer ranged from 85-100% (Table 11). Recoveries of HTSH from patient sera were found to frequently exceed 100% (Table 12). Using the same experimental data, the "HTSH found" was re-calculated using standard curves constructed from standards diluted in calf serum. As predicted, recoveries were lower approaching 100% (Table 13).

Accuracy was also estimated by analysis of quality-control samples (Beckman assay range 45-60 µIU/ml). The mean value obtained was 65.9 ± 2.5 (S.D.) µIU/ml. The coefficient of variation was 3.8%, (n=10). Using standards diluted in calf serum the mean value was 52 µIU/ml.

Clinical Results

HTSH assays were performed on fifty patient sera submitted for thyroxine (T_4), and T_3 uptake tests. In 36 sera having normal thyroid function tests the mean serum HTSH was 4.7 µIU/ml, (S.D. 1.2 µIU/ml). The range was found to be 2.5 - 8.2 µIU/ml. No sex differences could be demonstrated. The "expected mean" for the Beckman HTSH System was "approximately 10 µIU/ml" (23). Six sera having T_4 levels below normal (5.4 - 13 µg %) were tested. Four of these sera had elevated HTSH results. The HTSH concentration in patient sera having T_4 levels greater than normal ranged from 2.2-3.8 µIU/ml. A comparison of HTSH, T_4, T_3 uptake and free thyroxine index (F.T.I.) values obtained in patient sera is presented in Table 14. Previous studies indicate HTSH levels in hyperthyroid patients range from less than 1 µIU/ml to less

HTSH Added µIU/ml	HTSH Found µIU/ml	% Recovery
0	0	--
2	1.7	85
4	3.8	95
8	7.8	97.5
16	15.5	96.9
32	32	100
64	64	100
128	128	100

Table 11: Recovery of HTSH From Buffer

	SERUM A		SERUM B	
HTSH Added µIU/ml	HTSH Found µIU/ml	% Recovery	HTSH Found µIU/ml	% Recovery
0	18	--	2.6	--
2	19.5	75	4.9	115
4	21.5	87.5	6.6	100
8	26.5	106.3	11.6	112.5
16	35	106.3	23	127.5
32	55	115.6	44	129.4

Table 12: Recovery of HTSH From Patient Sera. HTSH Standards diluted in buffer.

	SERUM A		SERUM B	
HTSH Added µIU/ml	HTSH Found µIU/ml	% Recovery	HTSH Found µIU/ml	% Recovery
0	10	--	1.1	--
2	12	100	3.1	100
4	14.4	110	5.0	97.5
8	18.4	105	9.5	105
16	26	100	17	99.4
32	42.5	101.6	33	99.7

Table 13: Recovery of HTSH From Patient Sera. HTSH Standards diluted in calf serum.

Patient	HTSH, (μIU/ml)	T_4 (ug%)*	T_3 uptake (%)*	F.T.I.*
A	2.6	1.3	27	0.1
B	5.7	3.4	34	0.4
C	> 64	3.5	26	0.3
D	300	< 1	22	---
E	22	2.7	24	0.2
F	2.9	21.3	27	2.1
G	3.1	19	34	2.4
H	2.2	17.4	43	2.8
I	3.0	20.4	44	3.3
J	2.5	20.4	41	3.1
K	3.4	6.2	28	0.6
L	4.7	10.2	24	0.9
M	6.2	7.3	25	0.7
N	6.9	4.0	37	0.5
O	4.4	5.6	37	0.8
P	7.0	7.2	30	0.8
Q	4.8	4.4	38	0.6
R	5.1	6.5	24	0.6
S	4.0	8.4	30	0.9
T	5.5	12	21	0.9

*Lab Normals

Serum T_4	5.4 - 13 ug%
T_3 Uptake	25 - 35%
F.T.I.	0.5 - 1.7

Table 14: A Comparison of HTSH, T_4, T_3 uptake and Free Thyroxine Index values in Patient Sera

than 4.5 µIU/ml. In hypothyroidism HTSH values are usually elevated with values ranging from 20 µIU/ml to well over 500 µIU/ml (23).

Patient serum drawn before and after the intravenous injection of TRH (200 ug) was assayed using the Beckman procedure. A peak level of 19 µIU/ml was achieved in the twenty minute sample. The forty and sixty minute specimens contained 17 µIU/ml and 13 µIU/ml HTSH respectively. The basal level was 4.9 µIU/ml.

Correlation Study:

Thirty patient sera were analyzed in duplicate by the Beckman HTSH System and by a reference laboratory using a "Microliter Plate Solid Phase Radioimmunoassay for HTSH" (30). In the Correlation Study, Table 15, the results obtained are listed in ascending order of concentration. The bias was calculated and subtracted from the differences to separate the systemic bias from the inherent variability (13). The standard deviation of the differences minus the bias was calculated and the t test used to test the significance of the observed bias. The calculated standard deviation of the difference was ± 7.55 µIU/ml. The t value is 0.68. From the Table of t values at 95% limit, 27 degrees of freedom, the t value is 2.05.

The sign test was also used as an additional check for internal consistency of the data. Eleven minus signs and seventeen plus signs are observed in Table 15. According to the table value for sign testing, the minimal number of plus and minus signs for 28 specimens is 8. A negative bias is demonstrated at low HTSH levels. Examination of Figure 15 comparing the dose-response curves obtained for the two methods demonstrates that the Beckman HTSH System has greater sensitivity, detecting small dose levels of HTSH. The reference procedure could not estimate

Analysis	Solid Phase HTSH Results	Beckman System HTSH Results	Difference	Difference Minus Bias
1	2	3.3	-1.1	-2.07
2	2	4.7	-2.7	-3.67
3	2	3.9	-1.9	-2.87
4	2	3.8	-1.8	-2.77
5	3.6	4.1	-0.5	-1.47
6	4.9	4.2	+0.7	-0.27
7	5.0	4.6	+0.4	-0.57
8	5.4	4.5	+0.9	-0.07
9	6.1	5.0	+1.1	+0.13
10	6.1	4.0	+2.1	+1.13
11	6.9	5.7	+1.2	+0.23
12	7.7	4.9	+2.8	+1.83
13	8.0	5.2	+2.8	+1.83
14	8.2	4.5	+3.7	+2.73
15	9.2	5.1	+4.1	+3.13
16	9.4	4.5	+4.9	+3.93
17	9.4	3.5	+6.4	+5.43
18	9.6	3.4	+6.2	+5.23
19	9.7	4.0	+5.7	+4.73
20	10.1	8.2	+1.9	+0.93
21	18.0	8.6	+9.4	+8.43
22	18.1	13.0	+5.1	+4.13
23	18.5	22.0	-3.5	-4.47
24	18.8	17.0	+1.8	+0.83
25	20.5	54.0	-33.5	-34.47
26	27.7	19.0	+8.7	+7.73
27	67.0	69.0	-2	-2.97
28	115	110	+5	+4.03
29	158	>128		
30	300	>256		

Mean: Solid-Phase Method $\bar{X}$ 15.39 Beckman HTSH System $\bar{X}$ 14.42

Bias: Plus 0.97

Standard Deviation of the Differences ± 7.55

t test = 0.68; t value, 95% Limits. 27 d.f. = 2.05

Sign test: 11 minus signs, 17 plus signs
Sign Test Table for 28 specimens, minimal number + or - is 8.

Table 15: Correlation Study - HTSH Solid Phase RIA and Beckman Double Antibody System. Values in µIU/ml, Analysis of Serum.

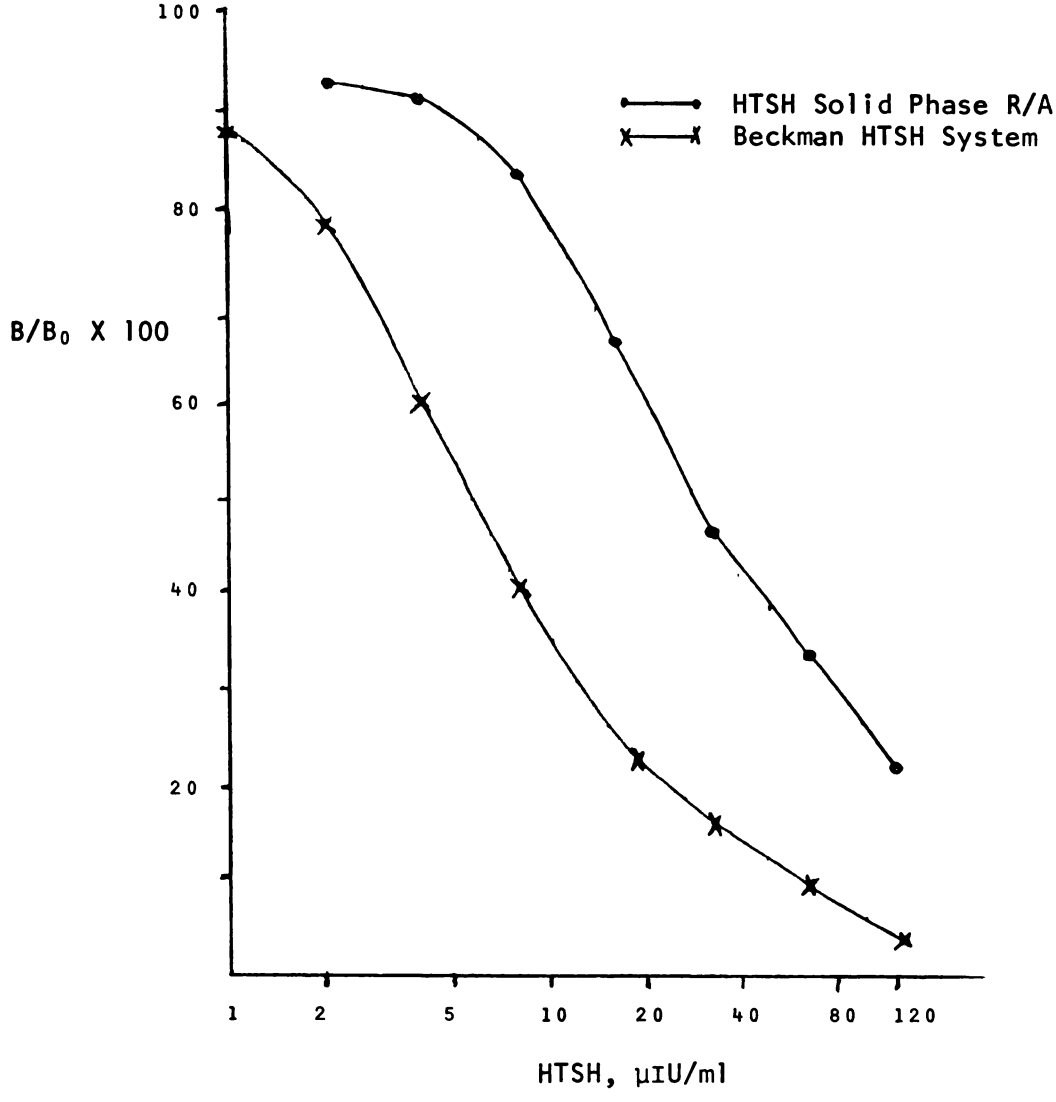


Figure 15: Comparison of Dose-response curves obtained using the Beckman Double Antibody System and a Solid Phase Microliter Plate Radioimmunoassay.

with confidence HTSH concentrations below 5 µIU/ml. The dose response curve for the Microliter Solid Phase Radioimmunoassay had a steeper slope at dose levels over 20µIU/ml HTSH.

The between assay reproducibility of the reference method (n=15) was ± 12.4% (17.7 ± 2.2 µIU/ml). The within assay precision (n=6) was calculated to be 6.7 ± 2.4 µIU/ml. At similar dose levels the between assay reproducibility for the Beckman System was 21.5 ± 1.8 µIU/ml (C.V. = 8.4%). The within-assay variability of the Beckman procedure was 5.4 ± 0.16 µIU/ml (C.V. = 3.0%).

GENERAL DISCUSSION

Acceptability of a method depends on both applicability and performance. Applicability encompasses factors such as sample size, types of samples useable, speed of analysis, equipment needed, personnel requirements, cost, and the like. Performance considers the type and magnitude of errors. The Beckman Twin-10 Test HTSH System tested under clinical laboratory conditions was found to provide a convenient reagent package which was easily introduced into laboratory use. Although no special skills were required, attention to cleanliness, an awareness of the possible sources of error, and consistency in technical procedure, were qualities of importance. Using the modified micro-assay protocol, a single technologist could assay up to thirty-five patient sera within 36-48 hours. This is appreciably faster than HTSH methods published in the literature (4, 9,11, 28). During the assay the technologist is available for other duties while the reaction tubes are incubating and while the radioactive counts are being collected. The cost of reagents using the micro-protocol was estimated to be approximately 95¢ per patient test. Using the Beckman protocol the reagent cost would be between $2.60 - $3.25 per patient sample depending on the number of standard curves included. It was found that serum or EDTA-plasma could be used. Samples stored at 4°C were found to be stable for at least one week and at -20°C for longer periods. Since a circadian rhythm has been reported for serum HTSH it is recommended that blood samples for HTSH determinations be collected when the TSH level has fallen to the daytime level (31, 32). The equipment and supplies needed for the HTSH assay were common with other radioimmunoassay procedures being carried out in the laboratory.

Three different lots of Beckman reagents were tested while evaluating the design and protocol of the HTSH assay. In their lyophilized form the Beckman reagents were found to be stable over long periods of time without loss of specificity, sensitivity or precision. The effect of freezing and thawing on the reconstituted reagents was also tested. Negligible change in percent binding or in the parameters of the dose-response curves was observed when the reconstituted reagents were stored at -20°C for up to six days.

For all of the reagent systems tested the desired level of counting accuracy (2σ = 2%) was achieved within 5-10 minutes. The binding affinity of the ^{125}I-HTSH was compared to that of the HTSH standard in assays containing different concentrations of binding reagent (Figure 16). The labelling process produced negligible change in the HTSH with respect to its binding of the antiserum. The stability of the labelled HTSH stored in its lyophilized form was demonstrated. For the reagent system tested, the percent bound at zero dose HTSH decreased only slightly from 20.5%B to 18.4%B over a period of four weeks. The total ^{125}I-counts collected over ten minutes fell from 91,000 to 69,500 during the same four week period. The difference in percent binding reported between the different reagent sets (Figure 9), suggests a difference in the concentration of reagents and/or in the immunoreactivity of the labelled hormones used. Good sensitivity was achieved with all labelled preparations employed.

The anti-HTSH antiserum was also found to be acceptable for clinical laboratory use. The sensitivity of the Beckman system and the results of the crossreaction studies provide evidence of satisfactory avidity,

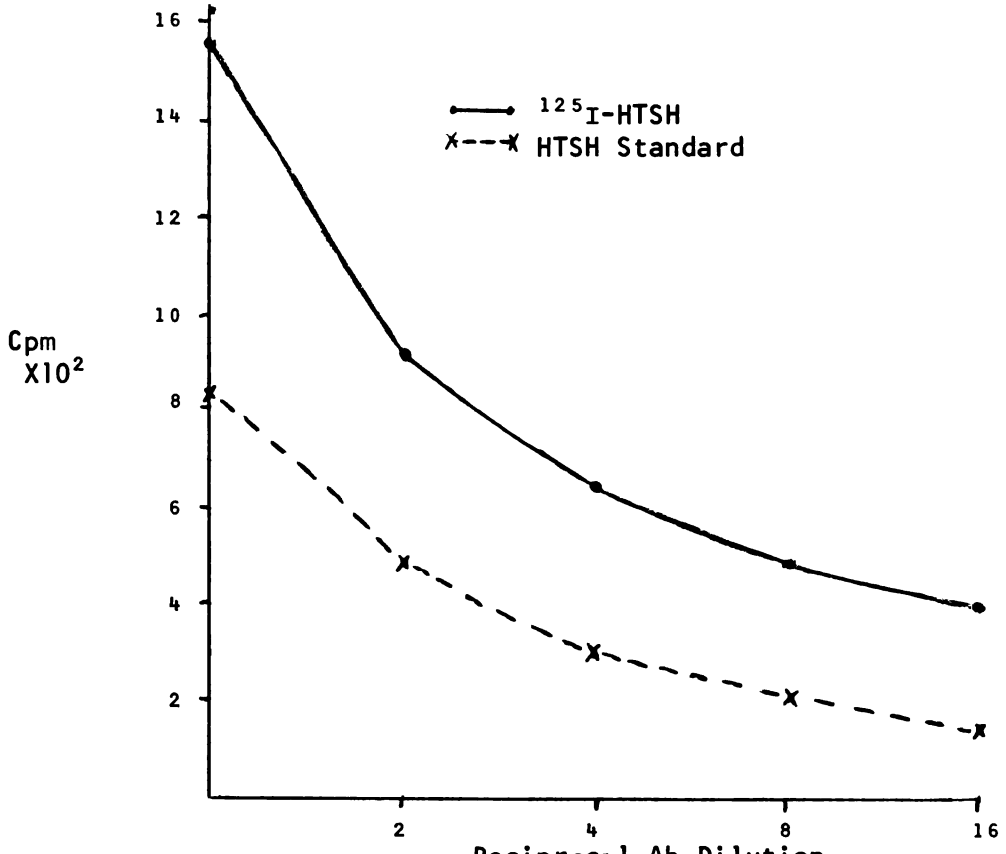


Figure 16: Comparison of binding energy of ^{125}I-labelled HTSH and HTSH standard.

specificity and titre. HCG levels over 30 I.U. would be unusual even in the serum of a pregnant woman (31). HCG levels up to 66 I.U. did not interfere with the recovery of HTSH. The cross reactivity of HLH and HFSH is reported to be approximately 1% and 2% by weight respectively (23).

The precipitating antibody when used in accordance with the Beckman instructions was sufficient to precipitate the primary antibody present in each reaction tube. Excess of either the primary or secondary (precipitating) antibody resulted in incomplete precipitation of the bound HTSH. The precipitating antibody was found to be stable several months in its lyophilized form. Valid results were also achieved when the reconstituted antibody was stored at -20°C for up to six days.

The reaction sequence and assay conditions recommended by Beckman provided optimal sensitivity and precision. Assay performance did not improve when the concentrations of antiserum and labelled antigen were reduced. Similarily altering the times and temperatures of the incubation periods did not improve the assay design. The method was found to be adaptable to a micro assay using 100 µl. of serum sample. The micro adaptation increased the number of samples which could be assayed, significantly reducing the reagent cost per test. With the exception of the "protein effect" the general reliability of the Beckman system was found to be excellent. Good precision was achieved at levels of HTSH important in the diagnosis of thyroid disease (Tables 7-10). It was found that HTSH levels over the range of 1.5 - 64 µIU/ml could be measured without diluting the serum. The accuracy of the method was determined using control sera with quoted HTSH concentrations. The between laboratory variation on 30 serum

samples assayed by a microliter solid phase procedure and the Beckman system was not significantly different.

The effects of protein on the standard curve was demonstrated after finding the recovery of HTSH from patient sera was unacceptably high. It is clear from Figure 11, that use of the buffer standard curve gives results significantly higher than when the standard curves employing the bovine γ globulin, canine or calf serum are used. As demonstrated in Table 13, using the standard curve containing calf serum the recoveries from patient sera were reduced such that they consistently approached 100%. For absolute accuracy it is necessary that the standards and patient sera be as identical as possible. It has been suggested that the protein content in each tube be kept equal by adding whole serum of different origin to the standards. Human thyrotoxic, human T_3-suppressed, human hypopituitary serum, dog, horse and bovine serum have all been used for this purpose (9). Sluiter (9) recommends the addition of a purified bovine γ-globulin solution to the standards. Bovine γ-globulin has the advantage that its concentration in the reaction tubes can easily be controlled and it is readily available in unlimited supply. The necessity of re-evaluating the assay system with the frequent introduction of new and different serum void of HTSH is also avoided. Sluiter has demonstrated that it is the globulin part of serum proteins which is responsible for the intervention in specific interactions in HTSH assays. This is consistent with the finding that the 1% and 5% BSA standard curves were similar to the Beckman curve diluted in phosphate buffer containing 1% BSA. The amount of bovine γ-globulin quantitatively simulating the effects of T_3-suppressed or hypopituitary serum is reported to be about 50 mg/ml (9).

The results obtained employing the Beckman system without increasing the protein content of the standards cannot be accepted as being "absolutely accurate." However since it is recognized that immunoassayable HTSH is generally not equivalent to biological TSH activity, the Beckman results can provide clinically useful and valid information in the diagnosis and treatment of thyroid disease. It is essential that the HTSH concentrations in normals and in individuals suffering thyroid disease be carefully and thoroughly studied. To facilitate comparison of the present data with that reported by other investigators, the mean and range of serum HTSH in euthyroid subjects is listed in Table 16 . When the standards for the assay are incubated in buffer alone the mean HTSH concentration is between 4.4 and 20.5 μIU/ml, with a range from <2 to >20.5 μIU/ml. Assays in which standards have been incubated in varying types of serum the results are significantly lower with the mean between 2.5 - 5.4 μIU/ml and individual values ranging from <1 to 10.4 μIU/ml. When thirty-six euthyroid sera were assayed by the Beckman HTSH system the mean value was 4.7 ± 1.2 (S.D.) μIU/ml, with a range of 2.5 - 8.2 μIU/ml. These findings are considerably lower than the published data of Beckman which suggests the mean of the normal range should be approximately 10 μIU/ml (23). The HTSH results obtained using the modified Beckman protocol (micro-assay) correlated well with the thyroid function tests reported in Table 14. The Beckman HTSH Reagent System fulfilled the main function of an HTSH assay in that it was able to identify sera from patients with hypothyroid activity. The HTSH results from sera having elevated T_4 levels supported a diagnosis of hyperthyroidism.

	Authors	HTSH, μIU/ml Mean	Range
STANDARDS IN BUFFER (low protein concentration)			
	Odell, 1968	4.4 males	2.8-9.0
		5.4 females	2.8-9.4
	McHardy-Young, 1969	20.5	±6.7, 1SD
	Sawin, 1970		<2 -12
	Hershman, 1970	4.4	
	Coble, 1970		3.6-7.3
	Lemarchand-Beraud, 1970	13.2	10-16
	Patel, 1971 (28)	5.2	1.8-8.0
	Beckman Inc., 1973 (23)	10	
STANDARDS CONTAINING SERUM PROTEINS			
	Raud + Odell, 1969	4.4 males	1.8-10
		5.4 females	
	Hall, 1970	4.8	<1 - 10.4
	Greenberg, 1970		<2-7
	Fleischer, 1970	2.5	
	Sluiter et. al. 1972 (9)	2.0	±1.53, 1SD

TABLE 16: Published normal values for immunoreactive serum HTSH assays (9, 23, 28)

CONCLUSION

The Beckman Twin 10-Test Reagent System, a commercial system available for the quantitative radioimmunoassay of human thyroid stimulating hormone (HTSH) has been evaluated for use in the clinical diagnostic laboratory. A micro adaptation of the Beckman protocol has been introduced increasing the number of patient assays possible per reagent package thus reducing the cost per sample. The assay system has proven to be simple, quick to perform and reliable. Experimental data has been offered indicating the Beckman HTSH System offers the level of accuracy, precision, sensitivity and specificity required for clinical use. The general reliability and acceptability of the system has been discussed. A description of the method, equipment and reagents employed has been presented.

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