CHAPTER III

MATERIALS AND METHODS

MATERIALS

-ANIMALS

Eleven 7-12 years old adult female cynomolgus monkeys (*Macaca fascicularis*), weighing 3.5-5.5 kg each, were caged individually in a light controlled (0600-1800 h) room with good ventilation. These monkeys obtained from the primate research unit, department of biology, Chulalongkorn University. They were fed daily with monkey chow (Pokphand Animal Feed Co. Ltd. Thailand) in the morning and supplemented with fresh fruit : pineapples, banana, sweet corn, melon, sweet potato including boiled eggs in the afternoon.

-PROTOCOL

These monkeys were divided into 2 groups as follow

(1) Experimental group (n=7)

In this group, the animals were force-fed daily with MMI in the dose of 10 mg/day by divided into two doses at 0800 and 1700 h. The experiment was started on D3 of cycle after observed previous normal consecutive 2

cycles. The first day of vaginal bleeding was designated D1 of the menstrual cycle and checked by vaginal swabbing. An initial dose of MMI was calculated from the dose of 30 mg/day given in hyperthyroidism patients and additional 5 folds increase in lower sensitivity to drug in the monkeys that earlier reported (McGinty and Wilson, 1949). When the monkeys become hypothyroidism by monitoring measurements of T3,T4,fT4 and TSH concentrations, 10 mg/day of MMI was reduced to 5 mg/day as maintenance dose. Subsequently, the drug was reduced again to 2.5 mg/day in order to elevate serum levels of thyroid hormones reaching the state of mild hypothyroidism. Finally, MMI was withdrawn and studied the hormonal changes for at least 100 days with fertility test. The post-treatment female monkeys were paired with fertile male for breeding on the eleventh through the fourteenth day of the cycle. The first day that found the sperm plug in vagina and confirmed by light microscope was counted to be Day 1 of pregnancy. Consequently, the vaginal bleeding during implantation was carefully observed which earlier reported in this colony. With regard to detection of the pregnancy, the abdominal palpation after 3 rd week of post-mating was performed and capable of estimating gestational age. In the case of successful pregnancy, thyroid hormones changes and related to TBG, E_2, P,TSH,and PRL were studied and compared with those in the normal pregnancy monkeys.

The protocol of blood withdrawal was weekly

collected during the early MMI treatment and severe hypothyroidism. In addition, intermittent blood withdrawal on D 5, 8, 10, 11, 12, 13, 14, 16, 18, 25, 29 and 34 of each cycle during the compensatory, mild hypothyroidism and recovery periods was performed. The experiment protocols and dose interventions of each monkeys were exhibited in figure 1-7.

The monkeys suffered from some disturbances such as loss of appetite, galactorrhea, myxedema and drowsiness during hypothyroidism were cared in the good conditions.

(2) Normal pregnancy group (n=4)

After 2 consecutive normal menstrual cycle, the fertile female monkeys were mated with fertile male monkeys on D11-D14 of cycles as the post-treatment fertility testing. Weekly withdrawal of blood samples was performed from D1 of pregnancy to the day at term and delivery.

-Blood Collection

Blood samples ranging from 4 to 6 ml were collected at 0900-1000 a.m. through femoral vein puncture in non-anesthesized monkeys. These samples were allowed to clot and serum was separated by centrifugation at 1000 g. (4 ^{0}C). Serum were then aliquoated and stored at -20 ^{0}C immediately. Since preventing repeated thaws, the samples must be aliquoated before freezing storage.

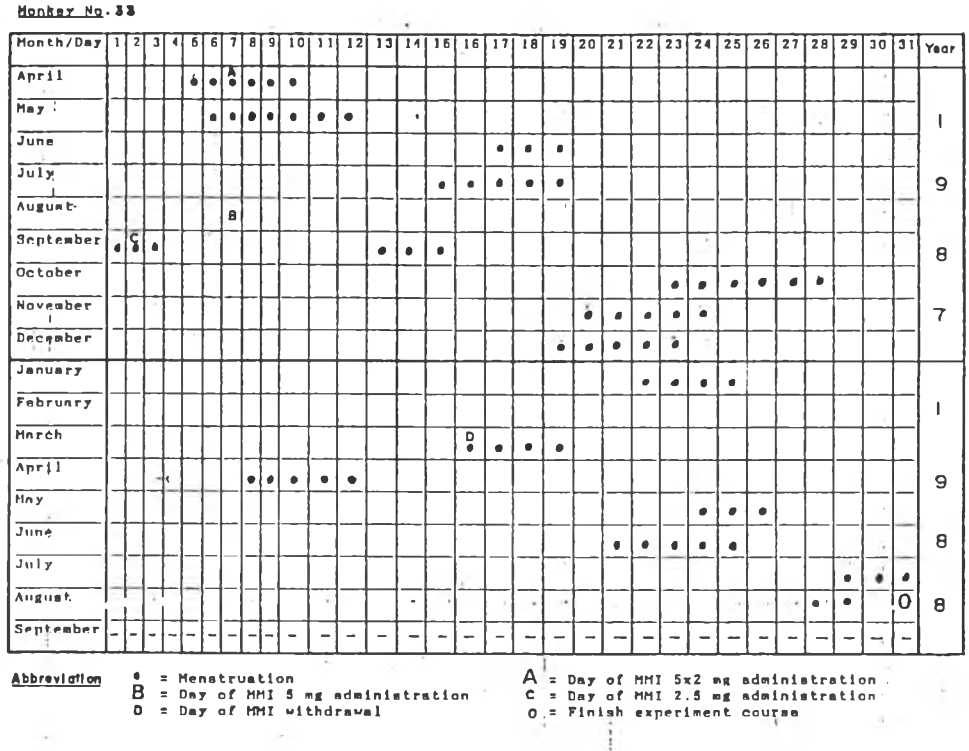

Fig 1. The studying protocol and menstrual cycle record in monkey no. 33. MMI 10 mg/day (A) was administered for 123 days and followed by 5 mg/day (B) and 2.5 mg/day (C) for 25 days and 195 days, respectively.

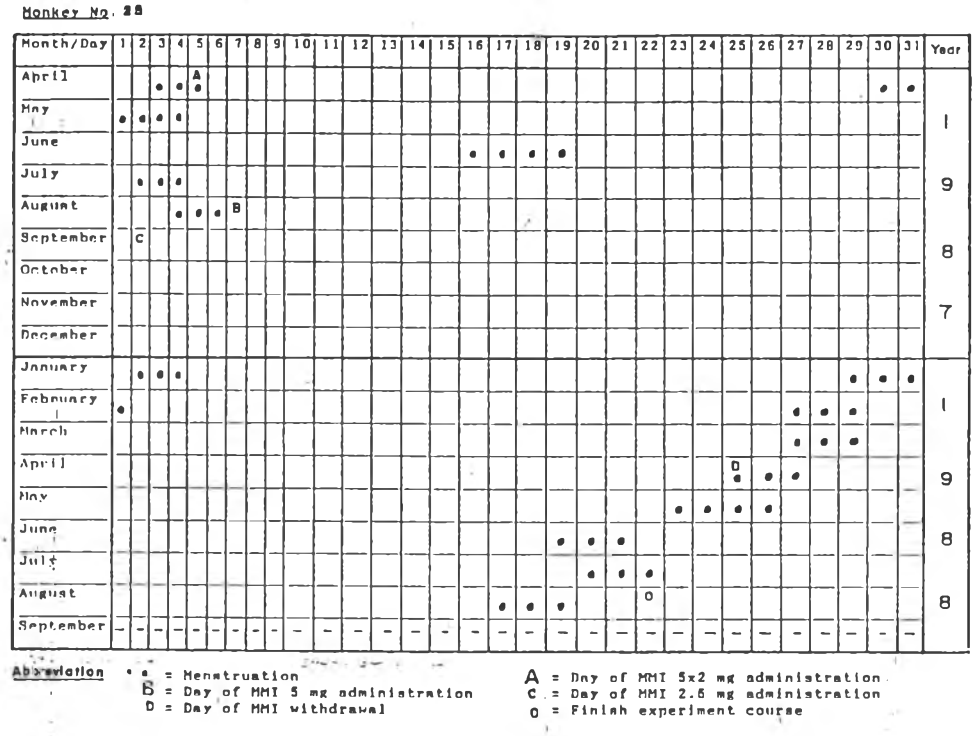

Fig 2. The studying protocol and menstrual cycle record in monkey no. 25. MMI 10 mg/day (A) was administered for 124 days and followed by 5 mg/day (B) and 2.5 mg/day (C) for 26 days and 235 days, respectively.

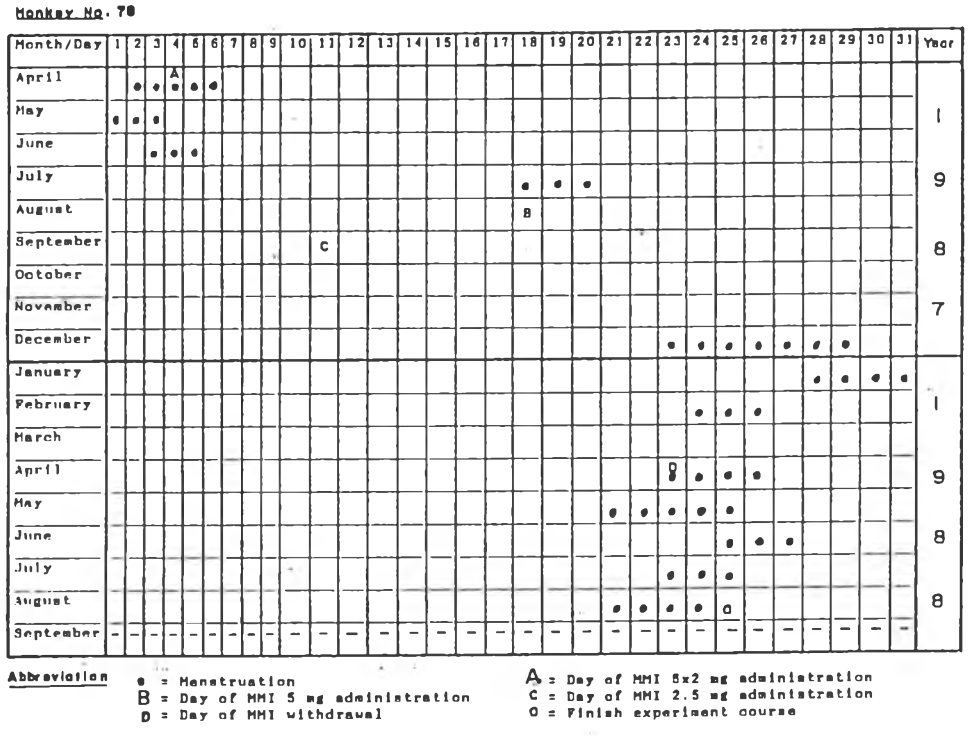

Fig 3. The studying protocol and menstrual cycle record in monkey no. 78. MMI 10 mg/day (A) was administered for 136 days and followed by 5 mg/day (B) and 2.5 mg/day (C) for 25 days and 226 days, respectively.

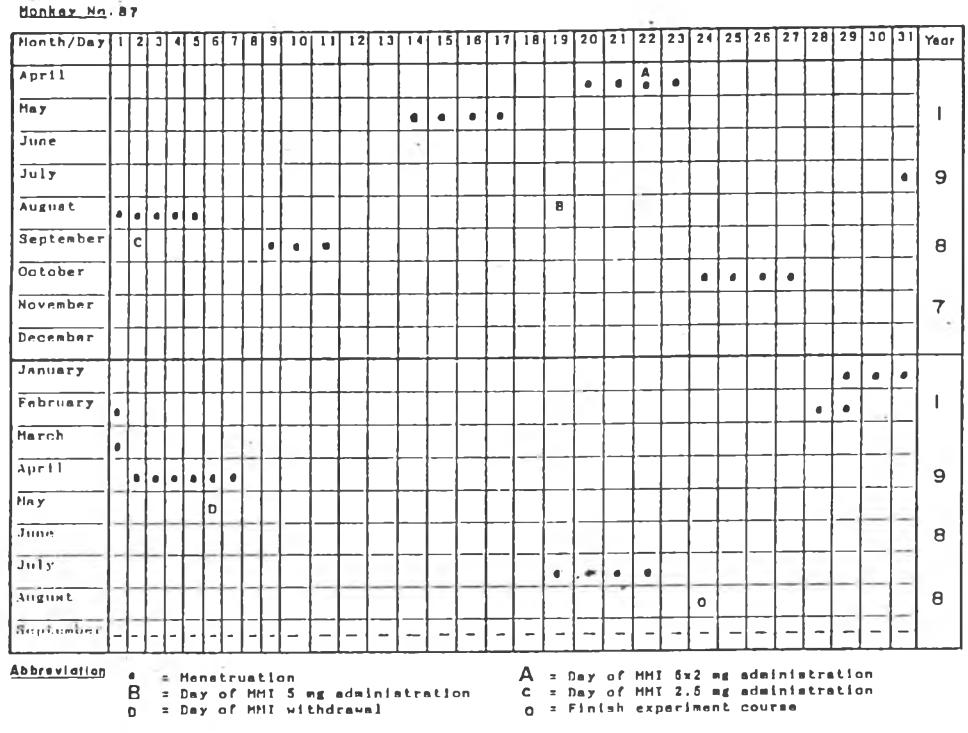

Fig 4. The studying protocol and menstrual cycle record in monkey no. 87. MMI 10 mg/day (A) was administered for 119 days and followed by 5 mg/day (B) and 2.5 mg/day (C) for 21 days and 240 days, respectively.

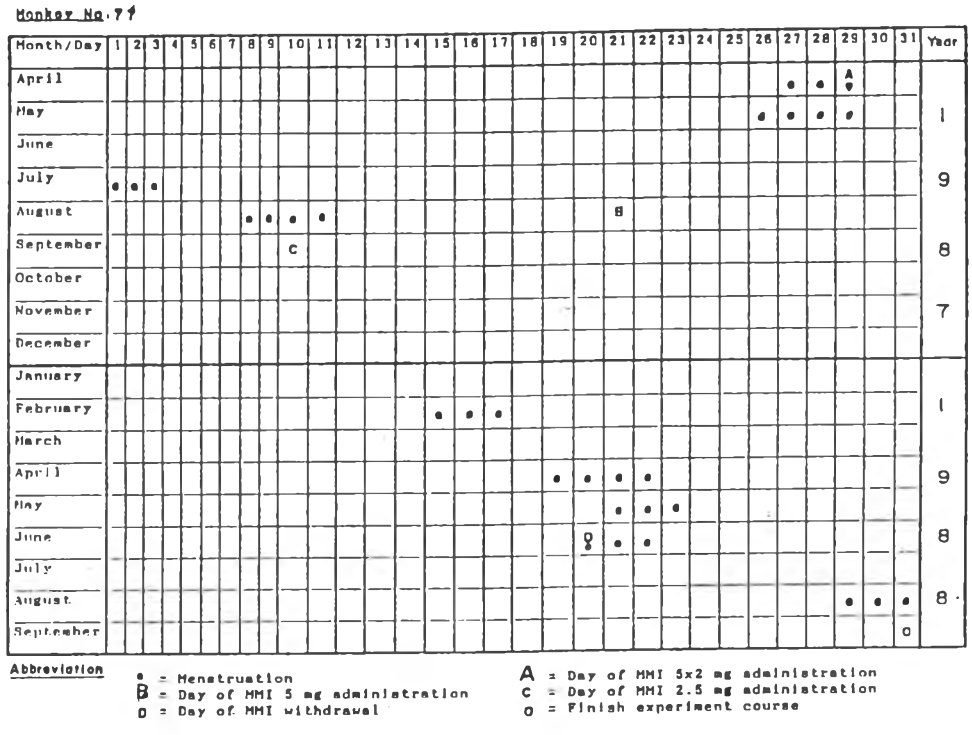

Fig 5. The studying protocol and menstrual cycle record in monkey no. 77. MMI 10 mg/day (A) was administered for 114 days and followed by 5 mg/day (B) and 2.5 mg/day (C) for 21 days and 284 days, respectively.

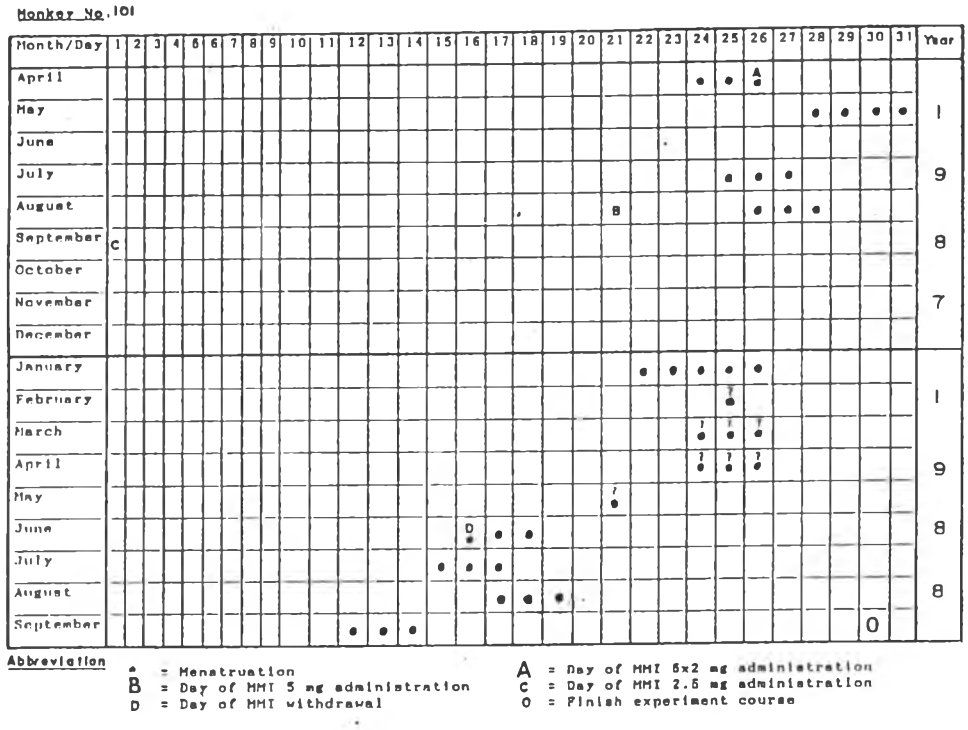

Fig 6. The studying protocol and menstrual cycle record in monkey no. 101. MMI 10 mg/day (A) was administered for 117 days and followed by 5 mg/day (B) and 2.5 mg/day (C) for 21 days and 279 days, respectively.

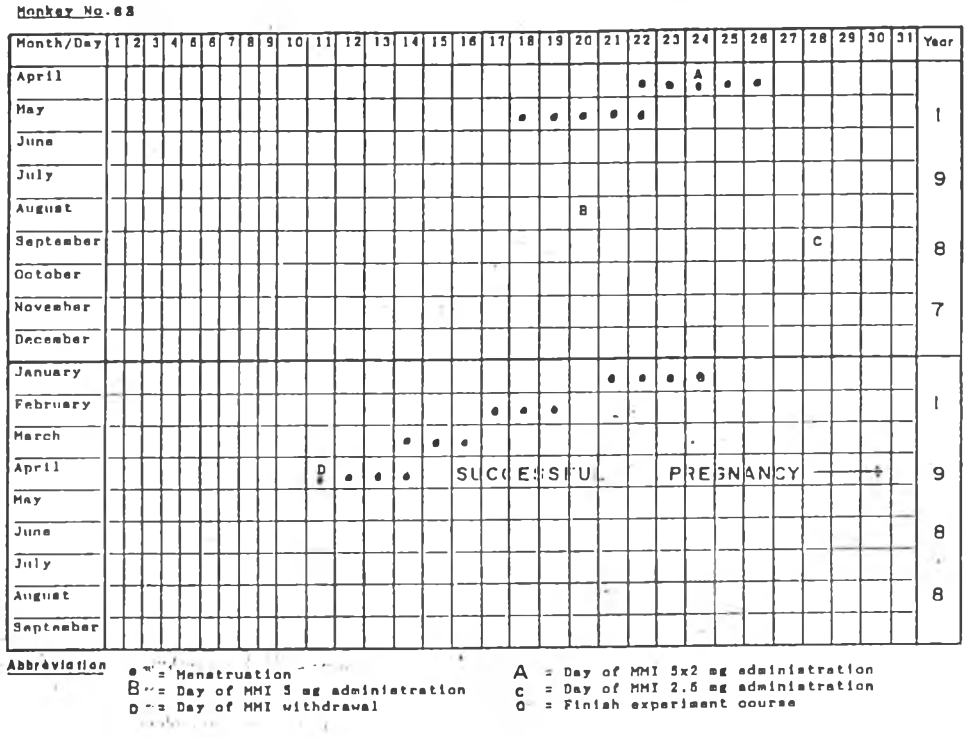

Fig 7. The studying protocol and menstrual cycle record in monkey no. 63. MMI 10 mg/day (A) was administered for 118 days and followed by 5 mg/day (B) and 2.5 mg/day (C) for 39 days and 196 days, respectively.

To prevent anemic states, all monkeys used in this study were given oral hematinics (Nutroplex liquiq^{R} : United American Pharmaceutical Inc. U.S.A.) throughout the conduction.

Methods

-Definitions (Ekins, 1978)

1. Sensitivity

The sensitivity of an assay system was minimal detectable dose determining from the zero dose intercepts of the standard deviation of precision profile.

2. Precision

Precision is refered to the less scatter of replicate measurements about the mean value arising from random errors. This may be expressed in terms of the CV or SD plotted against the amount or concentration of the substance measured. The precision of a measurement essentially defined the amount by which another measurement must differ for such a difference to be regarded as statistically significant. Intra sample, intra batch precision related to the scatter of replicate measurement on the same sample in the same assay batch. Inter sample, inter batch precision related to the scatter of measurements on different samples at the same dose level in the same assay batch.

3. Accuracy

Accuracy refered to the correctness of a measurement and expressed as the correlation coefficient between the determined and added values.

4. Working range

This constitutes the range of analyte concentrations which are measured with "acceptable" precision.

5. Specificity

This term related to the ability of an assay system to distinguish and measure the substance of interest and expressed as % cross reaction of 50 % of B/B_o.

I. Thyroxine(T4)

T4 measurement in cynomolgus monkeys serum was earlier performed by using human T4 kit (Smallrigde *et al.*,1981; Kamis, 1982;Ren *et al.*; 1988). In this study, commercial human T4 kit from Diagnostic Product Cooperation (USA) was used. Serial dilutions of cyn T4 concentrations were paralleled against human T4 standard curve (fig. 8). As with any heterozygous assay, values measured in this assay could not be interpreted as absolute values of the hormone. Therefore, the results were regarded only as relative values of the hormone. Furthermore, parallelism had also been interpreted as

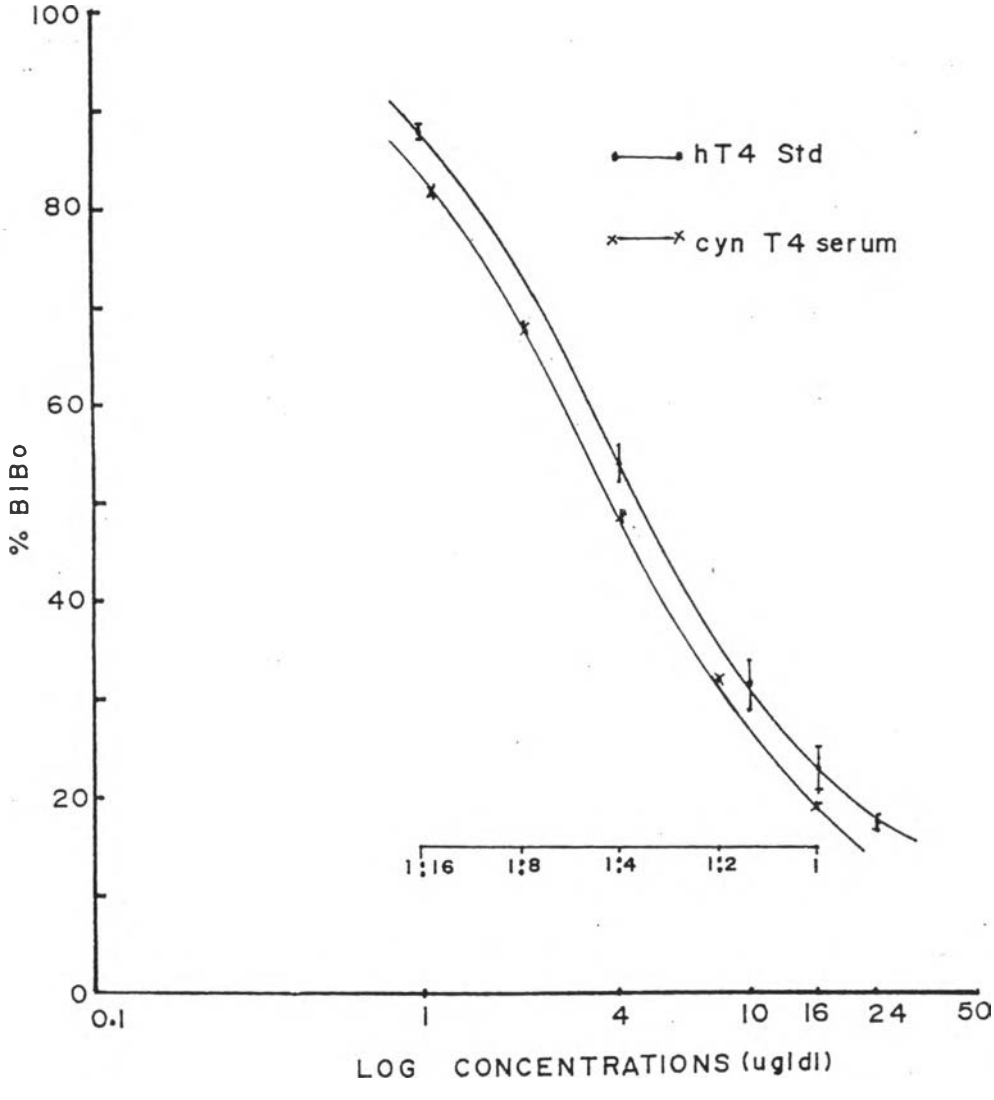

Fig 8. Paralellism in the immunoreactivities of human T4 standard and serial dilutions of cynomolgus T4 serum.

evidence of immunological similarity (Kieffer and Malarkey, 1978) or as suggesting that the observed immunoreactivity was not an assay artifact (Kyle *et al.*, 1987; Balfe, 1987). 150 ug of 8-anilino-L- naphthalene sulfonic acid (ANS) was used as a thyroid binding protein blocker whilst polyethylene glycol (PEG) as an acid to enhance the separation of bound from free forms (Chopra, 1972).

Reagents

1. T4 antiserum

Rabbit anti-human T4 store freeze dried at 4 ^{0}C was reconstituted with 50ml of distilled water and mixed by gentle inversion. This antiserum was stored at 2-8 ^{0}C for 30 days after reconstitution.

2. (^{125}I) Thyroxine

Each vial of ^{125}I-T4 in lyophilized form was reconstituted in 55 ml distilled water. The tracer was kept in refrigerator at 2-8 ^{0}C within 30 days after reconstitution. Prolonged storage of tracer should be required to re-column in order to get rid of free iodide.

3. Thyroxine Standard

The thyroxine standard were supplied in liquid form and contained the calibrators of 0, 1, 4, 10, 16 and 24 ug/dl which equivalently to 0, 12.9, 51.5, 129, 206 and

309 nmol/l. The human free T4 serum was designated the zero calibrator or blank. This calibrators were stored at 2-8 ^{0}C.

4. Goat anti-rabbit gamma globulin

This agent is stable if kept at 2-8 ^{0}C. Since a fine precipitate may form after refrigeration, the precipitating solution should be mixed throughly before use, without foaming.

The principle of assay

The procedure for T4 assay was a double-antibody RIA. ^{125}I-labeled T4 competed with T4 in the sample for antibody binding sites in the presence of ANS, the blocking agent, to prevent binding of radiolabeled T4 to thyroid hormone binding proteins. After incubation for 20 minutes,separation of bound from free form was achieved by the PEG-accelerated double-antibody method. Finally, the antibody-bound fraction was precipitated and counted in gamma counter. The concentration were read from a calibration curve consisting of x axis designated $\%B/B_0$ and y axis was the calibrator concentrations. Determining, B was the mean count (CPM) of any standard, unknown sample and quality control. B_0 or maximum binding was the total binding of (^{125}I) T4 and antiserum in the absence of competitive endogenous T4 or the standard in the assay tube.

T4 assay protocol

In 10 x 75 mm disposable polystylene tubes, the various reagents were added to yield a final volume of 600 ul. 10 ul of blank was pipetted into the blank and NSB tubes. Each remaining calibrator, quality control and unknown samples was aliquoated into the prepared tubes. 50 ul of (^{125}I) T4 was added to all tubes including total count (Tc) tube and shaked the rack briefly. Thereafter,50 ul of T4 antiserum was added to all tubes, except the NSB and Tc tubes, and vortexed. All were incubated for 20 minutes at room temperature. Precipitation of bound antiserum with 500 ul of cold second antiserum precipitating solution to all tubes except Tc tube and mixed well before incubated for 5 minutes at room temperature. Separation of bound form by centrifuged at 3000 g.,at 25 ^{0}C and for 15 minutes. The supernatant was decanted using a foam rack and let the tubes stood invertly on absorbent paper for at least 10 minutes. Then the tubes were trapped and blotted the rims in order to remove all residual droplets. The precipitate at the tube bottom was then count for 1 minute in gamma counter with the suitable window setting for I^{-125}.

Calculation

After recorded the CPM bound for each tube, the $\%B/B_0$ for each tube was calculated as :

$$\%B/B_0 = \frac{CPM_x - CPM_{NSB}}{CPM_{B0} - CPM_{NSB}} \times 100$$

where CPM_x = CPM for each standard, quality control,and unknown samples

CPM_{NSB} =Average CPM for the NSB tubes

CPM_{B0} = Average CPM for the blank tubes

Validation of T4 assay

1. Sensitivity

In T4 assay, the sensitivity was 0.20 ug/dl.

2. Precision

Three different of pooled cynomolgus monkey sera were made and used an assay internal quality control. High level quality control was collected from adult male monkey. Low and intermediated levels of quality control were pooled from hypothyroidism and normal female monkeys.

The within assay precision was evaluated by assaying 20 aliquoats of the same serum in the same assay, and represented as % coefficient of variation which calculated from means divided by standard variation of means as percentage. But the between assay precision was estimated by duplicate measurements in different assays on different days. A criterium for precision required less than a 20% coefficient of variation for both within and between assay variance (Hubl, 1980).

In T4 assay, the within-assay precisions were 5.20, 5.21 and 3.78% for low, intermediate and high T4 quality control samples. The % CVs of between-assay were 7.12% for low, 7.52% for intermediate and 3.87% for high pooled serum controls. The working range was 1.24-24.00 ug/dl at %CV 14.5-4.5.

3. Accuracy

The correlation coefficiency between determined and added values of T4 was 0.99.

4. Specificity

The cross reaction of T4 antiserum with other substances which may be presented in serum was tested by Diagnostic Products Corporation (DPC, USA) as showed in the following table. The cross reaction was calculated at 50% of B/B_0

Cross reaction substance	%cross reativity
L-thyroxine	100%
D-thyroxine	100%
Tetraiodothyroacetic acid	11.4%
Triiodo-L-thyronine	4.0%
Triiodo-D-thyronine	9.7%
Diiodo-L-tyrosine	0.06%
Monoiodotyrosine	0.36%
Methimazol	0.42%
6-n-Propyl-2-thiouracil	0.42%

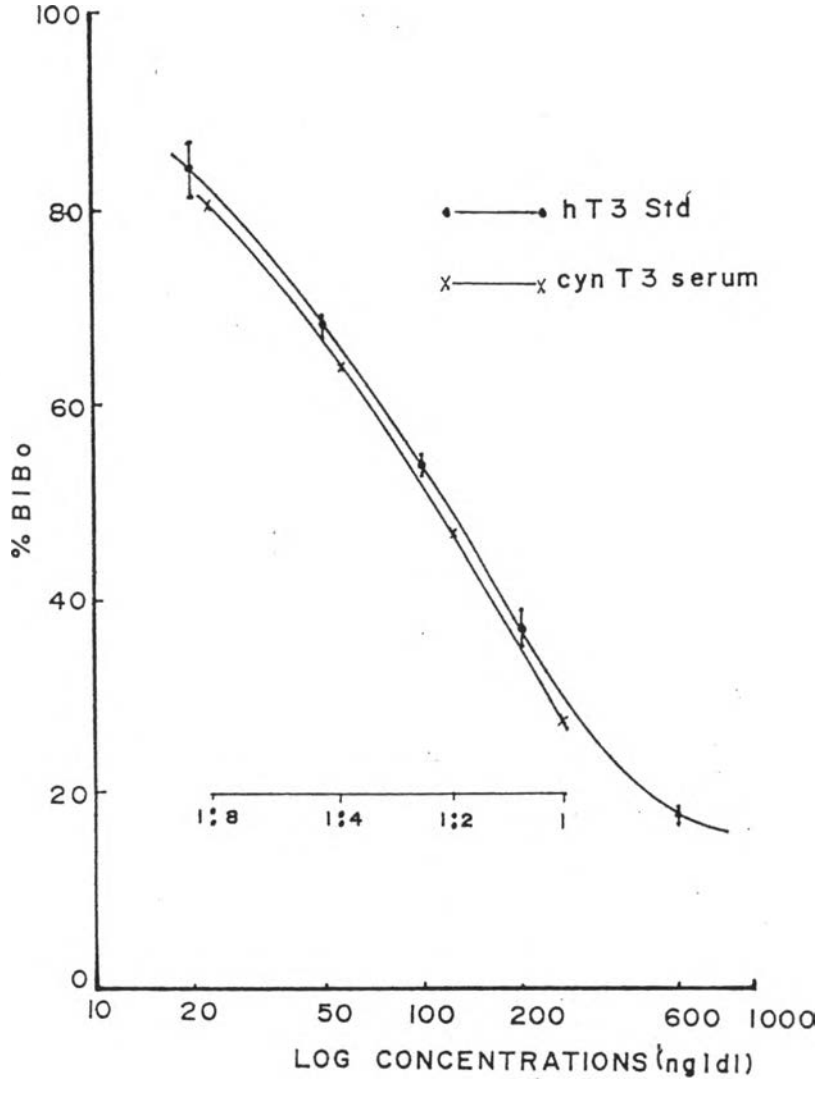

Fig 9. Paralellism in the immunoreactivities of human T3 standard and serial dilutions of cynomolgus T3 serum.

II. Triiodothyronine(T3)

T3 in cynomolgus monkey serum was also determined by commercial human T3 kit (DPC, USA) as previously reported (Smallridge *et al.*, 1981; Kamis, 1982; Ren *et al.*, 1988). The immunological similarity interpretation was performed by paralelled between human standard curve and serial dilution of cynomolgus serum (fig. 9).

Reagents

1. T3 antiserum

Rabbit anti-human T3 was provided in lyophilized form. This antiserum was reconstituted with 50 ml of distilled water and mixed by gentle inversion. After reconstitution, it is kept at 2-8 ^{0}C in refrigerator.

2. (^{125}I) T3

This tracer was provided in lyophilized form with ANS. It was reconstituted by adding 50 ml distilled water and mixed well, stored at 2-8 ^{0}C within 30 days.

3. T3 standard

Each T3 calibrators was provided in liquid form. Each vial contained the T3 standard in the concentration of 0, 20, 50, 100, 200 and 600 ng/dl which equivalently to 0, 0.31, 0.77, 1.54, 3.07 and 9.22 nmol/l.

4. Goat-anti rabbit gamma globulin

Each vial contained goat-anti rabbit gamma globulin diluted in PEG saline solution. This precipitating solution was kept at 2-8 ^{0}C. The precipitating solution should be throughly mixed without foaming prior to use.

T3 assay protocol

50 ul of duplicate standard, quality controls, NSB, B_0 and unknown serum was aliquoated to the labeled tubes. Radioiodinated T3 in quantitation of 50 ul was added to all tubes including Tc tube and mixed well. 50ul of T3 antiserum was then added to all assay tubes except Tc and NSB . All tubes were mixed immediately and incubated at room temperature for 60 minutes. Next step, 500 ul of the cold second precipitating antiserum was added to all tubes except Tc tubes and vortexed, re-incubated for 5 minutes at room temperature. All tubes except Tc tubes were then centrifuged at 3000 g (20 ^{0}C) for 15 minutes. The supernatant was decanted by using foam decanting rack. The precipitates left which represent of ^{125}I-T3 bound form were counted for 1 minute in gamma counter.

Validation of T3 assay

1. Sensitivity

The sensitivity of T3 assay was 5.20 ng/dl.

2. Precision

The within-assay of 10 pairs of the same sample was followed as : 9.17% for low level control, 4.62% for intermediate level control and 7.08% for high level control. %CV of between-assay of 20 different assays for low level was 9.84 %, for intermediate level of 5.73% and for high level of 9.12%. The working range of assay was 21-600 ng/dl at the level of %CV ranged 12.50-6.23%.

3. Accuracy

The correlation coefficient of T3 assay was 0.96.

4. Specificity

The cross reaction of T3 antiserum with other substances presented in serum was evaluated by DPC and reported as the following table.

Cross reaction substances	%Cross reaction
Triiodo-L-thyronine(T3)	100%
L-thyroxine(T4)	0.09%
D-Thyroxine	0.92%
Monoiodotyrosine	0.02%
Tetraiodothyroacetic acid	0.55%
3,5-Diiodo-L-tyrosine	0.03%
Methimazole	0.06%
6-n-Propyl-2-thiouracil	0.002%

III Thyroid stimulating hormone (TSH)

Cynomolgus TSH values in blood were relative values of human TSH (Smallridge *et al.*,1987; Ren *et al.*, 1988). The method exploited the ability of endogenous monkey TSH to inhibit competitively the binding of ^{125}I-labeled human TSH antibodies to human TSH as described by Wherry et al. (1970) and modified by Azukizawa et al. (1978). The dilution curves of a monkey pituitary homogenate or of plasma from the Rhesus monkey containing a high level of TSH were shared parallelism against the human TSH standard curve (Azukizawa *et al.*,1978; Melmed *et al.*,1979). In this assay, the high levels of cynomolgus TSH were paralleled against the human TSH standard curve as represented in fig 10.The monkey serum greater than 30 mIU/L was defined as high level in hypothyroidism and it was not necessary to diluted in the assay.

Reagent

1. Rabbit anti-human TSH

hTSH antiserum was provided in liquid form which consisted of normal rabbit serum, gelatin in barbital buffered saline and 0.02 M sodium azide as a preservative agent.

2. (^{125}I) TSH

Approximately 3 uCi tracer was in 5 ml gelatin in

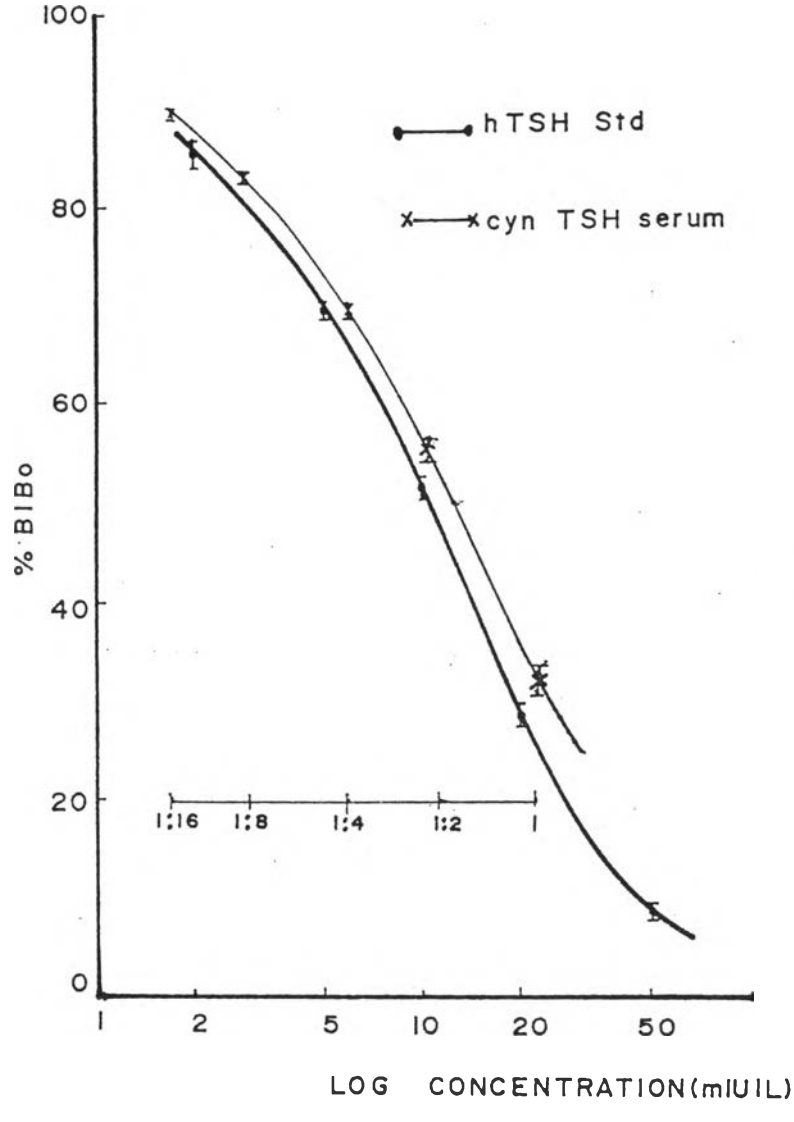

Fig 10. Paralellism in the immunoreactivities of human TSH standard and serial dilutions of cynomolgus TSH serum.

barbital buffered saline and 0.002 M sodium azide.

3. hTSH standard

The hTSH in this kit was calibrated against the Medical Research Council (MRC) World Health Organization, human pituitary TSH 68/38. The values for hTSH were expressed in terms of micro-international units per mililiter (uIU/ml) which converted directly to mIU/L of S.I. units. The hTSH standards were calibrated at 2, 5, 10, 20, 50 mIU/L in 2 ml of barbital buffered saline and BSA with 0.02 M sodium azide. This standards were kept at 2-8 ^{0}C.

4. Goat anti-rabbit serum (second antibody)

Goat anti-rabbit gamma globulin was in phosphate buffer with 0.02 M sodium azide. To insure a homogenous mixter, it was stirred continuously with a magneatic mixer and spin bar prior to use.

5. human TSH blank

Each vial contained 4 ml barbital buffered saline, BSA with 0.02 M sodium azide. Blank is calibrated at 0 mIU/L.

6. human TSH NSB reagent

Each vial contained 1 ml of normal rabbit serum, gelatin in barbital buffered saline and 0.02 M sodium

azide.

TSH assay protocol

200 ul of standards, quality control, unknowns was aliquoated to the duplicated tubes. 200 ul of blank was transfered to the NSB and B_0 tubes. In the NSB tubes, 100 ul of hTSH NSB solution was added. All tubes were then added with 100 ul of first antibody, rabbit anti-human TSH except Tc tubes and the NSB tubes and these were then mixed carefully. Thereafter, all tubes were incubated for 1 hr in a 37 ^{0}C water bath. 100 ul of (^{125}I) hTSH was added to all tubes including Tc tube and mixed well. They were further incubated for 2 hrs at 37 ^{0}C in the water bath. After that, 1000 ul of well-mixed precipitating antiserum solution was added to all tubes except Tc tubes and mixed, re-incubated for 15 minutes at 37 ^{0}C in water bath.They were separated by centrifugation at 1000 g,2-8^{0}C for 15 minutes. All tubes except Tc tubes were decanted and then counted for 1 minute by gamma counter with the suitable window setting for I-^{125}.

Validation of TSH assay

1. Sensitivity

In this assay, the sensitivity was 0.54 mIU/L.

2. Precision

The %CV of within-assay in low and high quality

controls were 11.72% and 6.77%, respectively. The %CV of between-assay was 14.84% for low quality control and 5.80% for high quality control. The working rang was 3.15-50 mIU/L at the level of %CV ranging 14.86%-4.12%.

3. Accuracy

The correlation coefficient of TSH was 0.99.

4. Specificity

The cross-reaction study was performed by Dade (Baxter travenol Diagnostics, USA) as the following :

Cross reaction substances	%Cross-reactivity
Thyroid-stimulating hormone	100
Luteinizing hormone	1.0
Follicle-stimulating hormone	<0.1
Human chorionic gonadotrophin	<0.1

IV Prolactin (PRL)

Cynomolgus serum PRL was measured in 100 ul of samples with a homologus human PRL RIA previously evaluated in rhesus monkeys (Quadri and Spies, 1976). Parallelism in immunoreactivities of human PRL standard and late pregnanted monkey serum was exhibited (fig. 11).

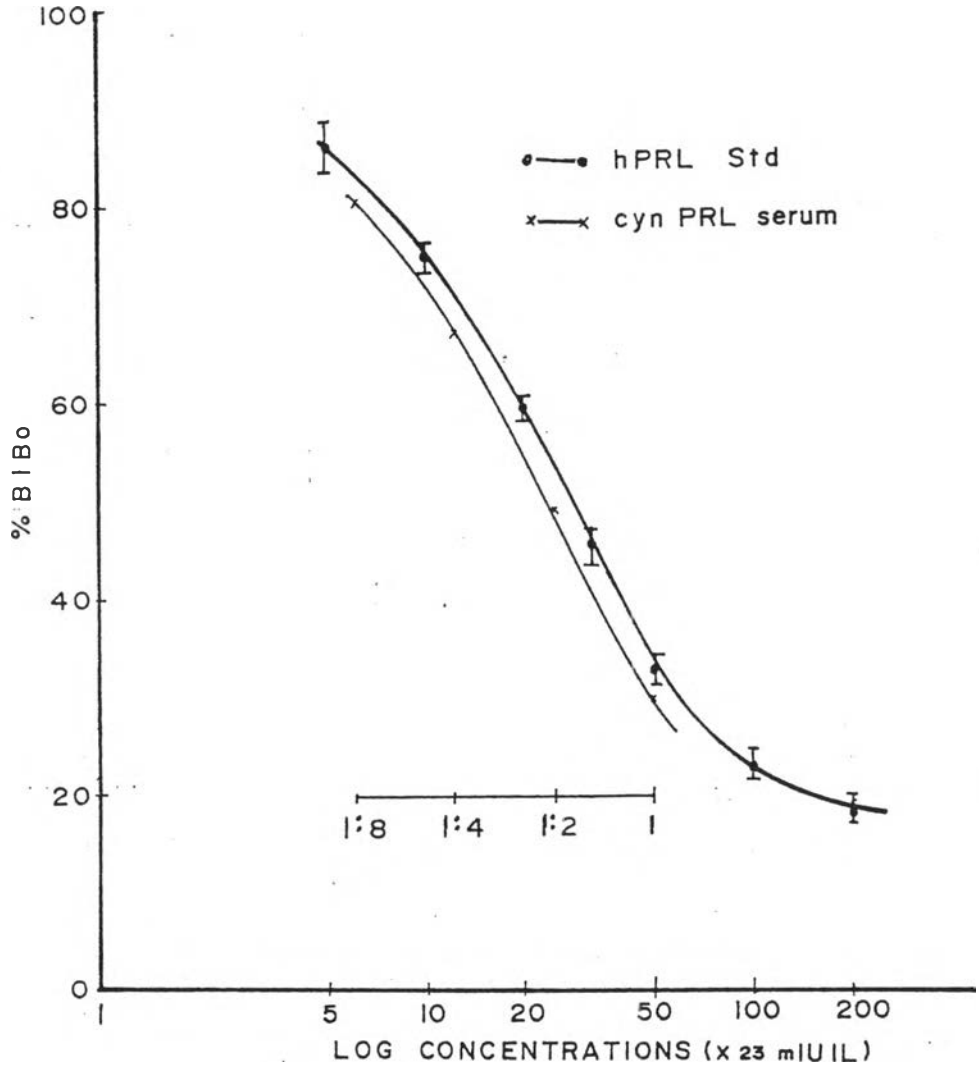

Fig 11. Paralellism in the immunoreactivities of human PRL standard and serial dilutions of cynomolgus PRL serum.

Reagents

1. Rabbit anti-human PRL serum

This first antibody was provided in a lyophilized form and to be reconstituted by adding 10 ml distilled water, mixed gently prior to use.

2. (^{125}I) PRL

The labeled human PRL was supplied also in a lyophilized form which had been purified by affinity chromatography. Each vial was reconstituted with 10 ml of distilled water and mixed well.

3. hPRL standard

The standards have been prepared in human serum stripped of PRL by affinity chromatography which represented 0, 5, 10, 20, 50, 100 and 200 ng/ml in terms of the WHO first international reference preparation of hPRL for RIA, no 75/504 (1st IRP 75/504). But in terms of the more recent second international standard for PRL, no 85/562, the standards have values of 0, 115, 230, 460, 1150, 2300 and 4600 mIU/L (2 nd 85/562).

4. Goat anti-rabbit gamma globulin (second antibody)

Each vial of second antibody were dissoveld in diluted PEG in saline. This precipitating solution must be kept in a cool place (2-8 ^{0}C) and should be throughly

mixed before use.

PRL assay protocol

100 ul of blank was pipetted to the NSB and B_0 tubes. 100 ul of each standards, quality control and unknown samples was aliquoated into prepared tubes. Iodinated human PRL 50 ul was then added to all tubes and mixed well. 50 ul of PRL antiserum was added later to all tubes except the NSB and Tc tubes. All were vortexed and incubated for 3 hours at room temperature. Consequently, 500 ul of cold, well mixed precipitating solution was added and vortexed. Separation of bound form was performed by centrifugation for 15 minutes at 3000 g, 20 ^{0}C. The supernatant was decanted and counted each tube for 1 minute.

Validation of PRL

1. Sensitivity

The sensitivity of this assay was 11.84 mIU/L.

2. Precision

The within-assay coefficients of variation were 7.78% for low level of quality control (Q.C.), 6.65% for intermediate level of Q.C. and 4.68% for high level of Q.C. . The between-assay precisions of low, intermediate and high levels of quality control were 6.16%, 7.41% and 12.98%, respsctively. The working range was 102.30-2148.50

mIU/L at the %CV 12.95-14.00%.

3. Accuracy

The correlation coefficient between the determined and added values was 0.97.

4. Specificity

The antiserum is highly specific for PRL, with an extremely low cross-reactivity to hGH, FSH, LH, TSH, hCG and hPL (DPC, USA).

V. Thyroxine binding globulin (TBG)

This method is based on sandwich ligand assay principles. TBG has many antigenic determinants due to its large molecule. Binding of anti-TBG antibodies to TBG usually does not interfere with the binding of T3 and T4 to TBG. Therefore, it is possible for TBG to bind to T4 and anti-TBG simultaneously (Chan and Aucock, 1980). The tracer, ^{125}I-T4, is added to both TBG standard and serum samples in the assay. Labeled T4, endogenous T4 from serum samples, and excess unlabeled T4 in the tracer form a T4 pool and form an equilibrium with the TBG in the serum samples. Since ^{125}I-T4 tracer is in a fixed amount per tube, the tracer which binds to TBG is proportional to the total TBG presents in the sample. Sufficient anti-TBG serum is then added to each assay tube to bind all the TBG.

TBG-bound tracer is separated from the remaining tracer pool by the anti-TBG antibody which forming the precipitate with the T4-TBG-anti-TBG complexs.The antibody bound tracer, located in the precipitate , is counted for radioactivity. The cyn TBG was measured and based on this principle using hTBG kit (Baxter Travenol,USA). Serial dilution of cyn TBG concentration paralled against hTBG standard curve was shown (fig. 12).

Reagent

1. Sheep anti-TBG serum

Sheep anti-TBG serum was in phosphate buffered saline, normal goat serum and 0.02 M sodium azide.

2. (^{125}I) T4 tracer

Approximately 7 uCi of iodinated T4 was in phosphate buffered saline, BSA and 0.02 M sodium azide.

3. TBG standard (1:41 dilution)

TBG was provided in 1 ml of processed human serum and gelatin in phosphate buffered saline with 0.02 M sodium azide at concentration of 5, 10, 25 and 50 ug/ml, respectively.

4. Second antibody (precipitating antiserum reagent)

Equine anti-goat serum was in phosphate buffer with 0.02 M sodium azide.

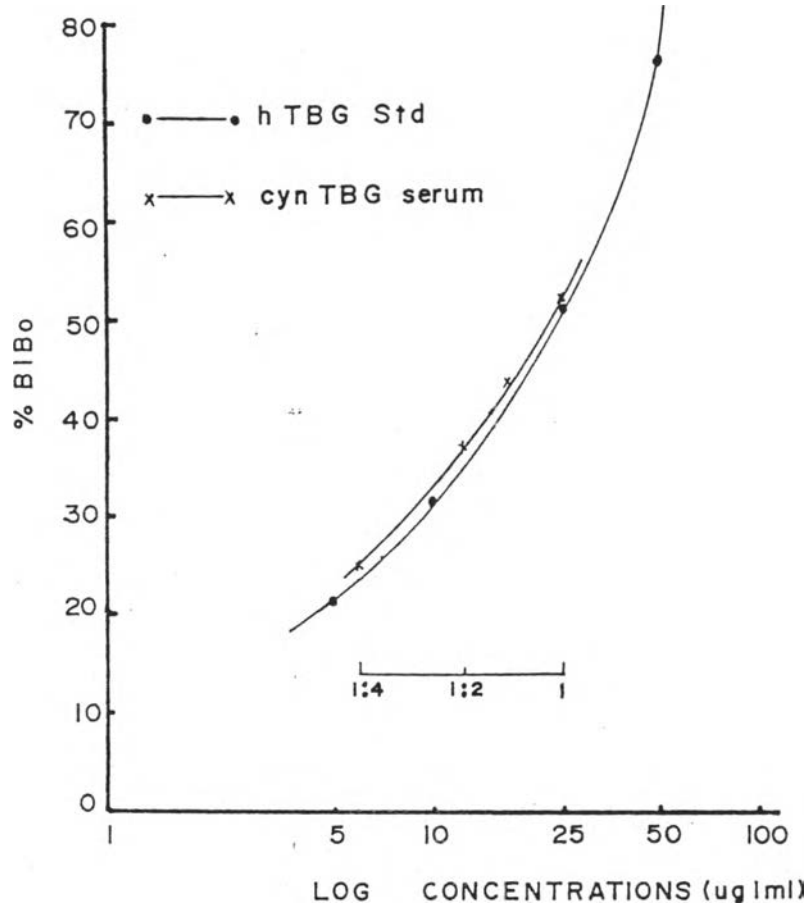

Fig 12. Paralellism in the immunoreactivities of human TBG standard and serial dilutions of cynomolgus TBG serum

5. TBG serum blank, 1:41 dilution

TBG serum blank was provided in a 1 ml of processed human serum and gelatin in phosphate buffered saline with 0.02 M sodium azide. Blank was calibrated at 0 ug/ml.

TBG assay protocol

Before assay,both the unknown and quality control samples were diluted 1:41 with assay buffer. The volume of 50 ul of TBG blank, standards, diluted quality controls and unknown samples were aliquaoted in the duplicates and transfered to assay tubes. One hundred ul of iodinated T4 was added to all tubes and followed by 100 ul of sheep anti-TBG serum. All tubes except Tc tubes were mixed by gently shaking of the test tube rack. This was then incubated for 10 minutes at room temperature. Consequently, 100 ul of precipitating antiserum reagent was then added to all tubes except Tc tubes and mixed each tube on a vortex mixer set at a low speed. All tubes were re-incubated for 10 minutes at room temperature. Separation of bound from free form was performed by centrifuged at 1000 g (25 ^{0}C) for 15 minutes, decanted,and counted in gamma counter thereafter.

Validation of TBG

1. Sensitivity

In this assay, the sensitivity was 1.1 ug/ml.

2. Precision

The within-assay of % coefficient variation performed in the intermediate and the high quality controls taken pooled sera from normal and pregnant monkeys was 2.55% and 5.16% respectively. The %CV of between-assay was 5.68% for the intermediate level and 8.63% for the high level. The working range was 3.78-50 ug/ml at %CV of 14.2-2.02%

3. Accuracy

The correlation coefficient between the determined and added values was 0.99.

4. Specificity

Data on the cross-reactivity of the antiserum used in this kit to various substances are shown in the following table. Varying levels of potential cross-reactants were added to a serum pool. Results, expressed as percent recovery, were obtained by comparing TBG values before and after spiking.

Compound	Amount Added(ug/ml)	TBG value (ug/ml)	%Recovery*
-Thyroxine(T4)	0	17.5	-
	1	17.5	100
	4	17.8	102
	8	19.0	109
	12	16.8	96
	20	17.6	101
-Aspirin	0	23.8	-
	2	25.8	108
	20	26.0	109
	200	24.0	101
-Valium	0	23.8	-
	1	25.0	105
	10	23.5	99
	100	22.9	96

VI Free thyroxine (fT4)

Cynomolgus fT4 was determined by two step "immunoextraction" procedure which has shown to correlate most closely with accepted reference method of equilibrium dialysis under various clinical conditions that earlier reported by Ellis and Ekins (1975) and Wirquin *et al.* (1987). Parallelism between cyn fT4 serial dilution curve and hfT4 standard curve was exhibited (fig.13) The two step assay (Dade,Baxter Travenol Inc., Cambridge, MS) was

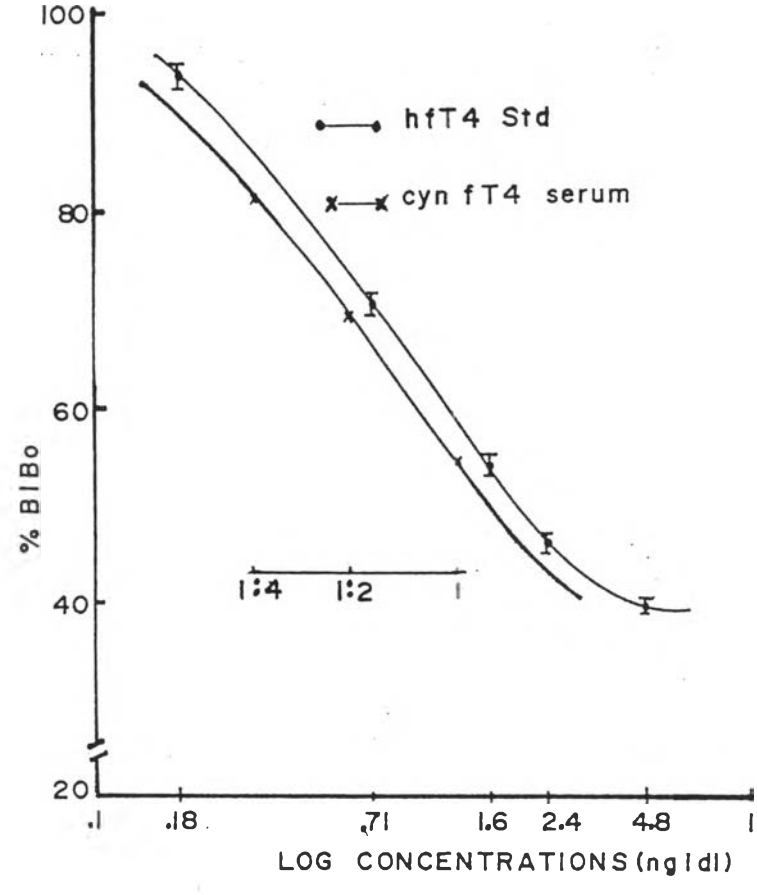

Fig 13. Parallelism in the immunoreactivities of human fT4 standard and serial dilutions of cynomolgus fT4 serum.

divided into 2 parts for removal of TBG-bound T4 from the assay tube.

Principles of the methods

The Gammacoat^{TM} kit for fT4 was performed entirely in the coated tube. During the first incubation, the fT4 fraction in each sample and standard was bound to the T4 specific antibody immobilized on the lower inner wall of the Gammacoat^{TM} tube. The T4 fraction which remained in solution bound to serum binding proteins was removed from the coated tube by decantation. The tubes were rinsed once with incubating buffer and then re-incubated with T4 tracer. The tubes were decanted again and counted.

Reagents

1. Rabbit anti-T4 serum coated tube

Polypropylene tubes (12x75 mm) coated with rabbit-anti-T4 serum were provided.

2. (^{125}I) thyroxine (T4) tracer

Approximately 5 uCi was in 10 ml of tris buffered saline with 0.02 M sodium azide.

3. T4 serum standards

Each vial contained L-thyroxine in 1 ml of processed human serum with 0.02 M sodium azide. Standard were calibrated at concentrations of 0.21, 0.88, 1.90,

3.00 and 5.80 ng/dl.

4. T4 serum blank

Each vial contained 1 ml of processed human serum with 0.02 M sodium azide. Blank was calibrated at 0 ng/dl.

5. T4 reagent buffer concentrate

Each vial contained 10 ml of tris buffered saline, 4 mM ANS, 6 mM sodium salicylate with 0.03 M sodium azide.

6. Incubation buffer

Each vial contained 125 ml of tris buffered saline with 0.02 M sodium azide.

Reagent preparation

-Tracer-buffer reagent

One vial of T4 reagent buffer concentration (10ml) as well as the entire content of T4 tracer (10 ml) were added and mixed well. 90 ml distilled water was then added and mixed gently. This tracer-buffer reagent must be stored in the dark or foil wrapped container, at 2-8 ^{0}C in refrigerator.

Free T4 assay protocol

50 ul of serum blank, each T4 serum standard, quality controls and unknowns was then added to the bottom

of the duplicate tubes. 1000 ul of incubation buffer was added to all tubes except Tc tubes and shaked the test tube rack. All tube except Tc tubes were incubated for 20 minutes in a 37 ^{0}C water bath. Consequently, all tubes (except Tc tubes) were decanted and allowed the tubes to drain in an inverted position for 3-5 minutes. The tubes were trapped on absorbent paper to remove any adhering liquid before placing the tubes upright. Incubation buffer in 1000 ul was then added to the each tube except Tc tubes and then decanted. This second addition of incubation buffer assured removal of TBG-bound T4 from the assay tube. All tubes were added with 1000 ul of tracer-buffer reagant, shaked gently, and re-incubated for 60 minutes at room temperature. All tubes were decanted except Tc tubes and then counted in a gamma counter for 1 minute with the window suitably adjusted for iodine-125.

Validation of fT4

1. Sensitivity

The sensitivity of this two-step assay was 0.02 ng/dl.

2. Precision

The percentage of coefficient variation in the within-assay was 12.50% for the low quality control pool, 6.25% for the intermadiate quality control pool and 12.82% for the high quality control pool. In the between-assay, the

%CVs were exhibited 14.35%, 6.89% and 16.38% for low, intermediate and high quality controls, respectively. The working range was 0.22-5.80 ng/dl at %CV 14.78-8.50%.

3. Accuracy

The correlation coefficient of fT4 was 0.99.

4. Specificity

Data in the cross-reactivity of the antiserum used in this kit were expressed as the ratio of the T4 concentration to the cross-reacting substance concentration at 50% inhibition of the maximum binding (Baxter Travenol,USA).

Cross-reactivity substance	%Cross-reactivity
L-thyroxine	100
D-thyroxine	100
L-triiodothyronine	2.6
D-triiodothyronine	2.1
D,L-diiodothyronine	0.1
L-diiodothyronine	0.1
D,L-monoiodothyronine	<0.1
L-monoiodothyronine	<0.1
D,L-tyrosine	<0.1

VII Luteinizing Hormone

The rhesus gonadotrophins do not cross-react in radioimmunoassay systems for the human gonadotrophic hormones (Neill *et al.*, 1976). In this assay, the LH kit consisting of purified cynomolgus pituitary LH (cyn LH), rhesus pituitary LH reference preparation and rabbit antiserum to hCG was kindly donated by National Institutes of Health (USA),through the National Pituitary Agency of the National Institutes of Arthritis Metabolic and Digestive Disease (NIAMDD).

Reagents

1. Rabbit antiserum to hCG (R13, pool D

Each vial contained 0.55 ml of undiluted antiserum.

2. Purified cynomolgus pituitary Lh (cyn LH) for radioiodination

Each vial contained 100 ul of purified cyn LH. The concentration was 0.15 ug/ml.

3. Rhesus pituitary LH reference preparation (NICHD-rhLH; also known as WP-XV-20)

Each vial contained 40 ug of rhLH standard. Since the purity of this preparation was approximately 10%, each vial's content corresponded to about 4 ug of pure rhLH.

Radioiodination of cynomolgus LH

Highly purified cynomolgus LH was iodinated by using chloramine T method as described by Greenwood and Hunter (1963).

Radioiodination is the process of introducing atom of radioactive iodine into a molecule. Iodine (either ^{125}-I or ^{131}-I) is generally introduced into the reaction mixture in the form of its sodium salt. While iodine can be incorporated into histidine and tryptophan residuces at pH 7-pH7.5 (those most commonly used for radioiodination) in a limited extent. Most iodine produced would be incorporated into tyrosine. The reaction that is belived to occur is oxidation of sodium iodide to form the iodous ion (I^+), an electrophilic agent. At alkaline pH the ortho position of the aromatic ring is activated for electrophilic attack due to the electron donating effect of the neighbouring hydroxyl group.

In addition, proteins can be damaged by the oxidaton of methionine and tryptophan residues during the iodination procedure. The effect of such oxidation damage on the physical and chemical properties of a protein or peptide cannot be predicted but can sometimes result in the loss of biological and immunological activity (Hunter, 1974).

Protocol

1.5 ug of purified cynLH was combined with 0.5 mCi of Na ^{125} I and 8.75 ug of chloramine T in a total reaction volume of 40 ul. The reaction was stopped 30 second later by the addition of 50 ul of solution containing 100 ug of sodium metabisulfite. After the addition of carrier protein (100 ul of 1% BSA in PBS plus 100 ul of gel-PBS), the reaction mixture is chromatographed on a small column (10 ml) of sephadex G-100 which has been equilibrated with gel-PBS. Fractions of 0.5 ml were collected and the radioactivity in each fraction was determined by survey meter after eluted with 0.1% gel-PBS. The pattern of radioactivity eluted should consisted of 2 peaks. The first peak (~ tube 5-16) was the radioactivity associated with the protein fraction. The second (tube 17-30) was associated with the salt fraction which consisted in part of the free iodine salt. Withdrawal of 10 ul from each tube collected and added to 12 x 75 mm disposable tube for counting.

This present iodination of cyn LH was showed the 32.3% yielding with 71.8 uCi/ug of specific activity. The iodinated cyn LH was stored in 0.1% gel-PBS, pH 7.0 and diluted with gel-PBS giving total count 0.1 ng/100 ul of 0.1% gel-PBS (~ 30,000 CPM). Four fractions were collected and tested for the maximum binding as following (fig.14)

Fraction no. 12, the % Bo = 10.23%

Fraction no. 13, the % Bo = 14.97%

Fraction no. 14, the % Bo = 20.45%

Fraction no. 15, the % Bo = 10.36%

Both fraction no. 13 and no. 14 were combined and showed the 18% of % maximum binding.

Preparation of Reagents

1. Anti-hCG R13

This first antibody was diluted 1:2000 in two steps.

1.1 The raw antiserum was diluted 1:50 with 0.05 EDTA in PBS (EDTA-PBS)

1.2 This 1:50 dilution was then further diluted with a 1:400 NRS in EDTA-PBS giving the working dilution 1:2000. At a final dilution of 1:10,000, 22% of the tracer was bound and this was the dilution which chosen for this assay.

2. Anti-rabbit gamma globulin

1 ml of anti-rabbit gamma globulin was diluted with 39 ml of 0.05 M EDTA in PBS.

3. RhLH standard (WP-XV-20)

The stock standard concentration was 1.0 ug/ml and frozen in -40^{o}C. The stock standard was thawed and then added 1.5 ml of 0.1% gel-PBS given the concentration of 1

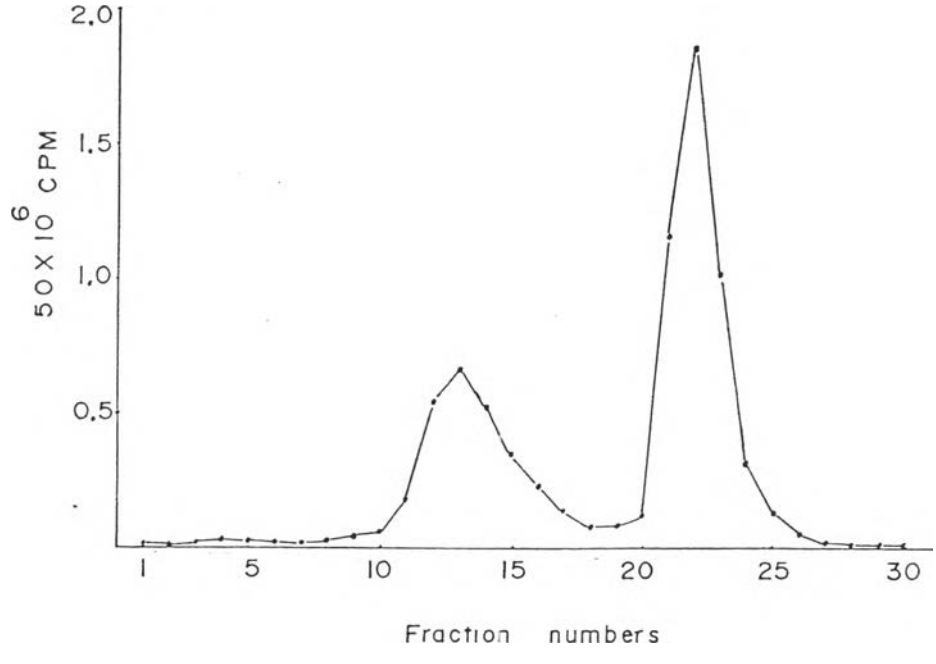

Fig 14. Iodination of mLH and fractional collection.

ug/2.5 ml or 80 ng/200 ul. This 80 ng/200 ul of standard LH was designated as a starting solution and sequently diluted with 1.0 ml of 0.1% gel-PBS. Then, the further six serial dilutions within a range of 1.25 - 40 ng/200 ul are made from this.

Cyn LH RIA protocol

Day 1 : 200 ul of first antibody was added to 200 ul of duplicate standard, quality control and unknown. All made up the total volume with 500 ul of 1% BSA-PBS. All tubes were then vortexed and incubated for 20-24 hrs at $4^{o}C$.

Day 2 : 100 ul of iodinated cyn LH was added to all tubes including Tc tube and then mixed, incubated for 20-24 hrs at $4^{o}C$.

Day 3 : The second antibody was added to all tubes except Tc tubes in volume of 200 ul, mixed and further incubated for 6 hrs at $4^{o}C$. Consequently, 3.0 ml of cold PBS was added to all tubes except Tc tubes. All tubes (except Tc tubes) were centrifuged at 1000 g, $4^{o}C$ for 30 minutes. unbound fraction was then carefully decanted. The precipitated bound form was counted in a gamma counter for 1 minute. This procedure was modified from Monroe *et al.*, 1970 and summarized in the following table.

I.D.	Std./Q.C/ Unks (ul)	1% BSA-PBS (ul)	Antiserum (ul)	A CynLH* (ul)	B anti-gamma globulin	C Cold PBS (ml)
Tc	-	-	-	100	-	-
NSB	-	500	-	100	200	3.0
Bo	-	300	200	100	200	3.0
Std	200	100	200	100	200	3.0
Q.C.	200	100	200	100	200	3.0
Unks	200	100	200	100	200	3.0

A = incubate 20-24 hrs at $4^{o}C$

B = incubate 20-24 hrs at $4^{o}C$

C = incubate 6 hrs at $4^{o}C$

Validation of LH

1. Sensitivity

The sensitivity of this assay was 10 ng/ml

2. Precision

Only within-assay was performed for 5 replicates and showed the % CV of 4.40.

3. Accuracy

The correlation coefficient between the determined and added values of LH was 0.98.

4. Specificity

Detailed specificity studies with cyn LH : anti-hCG R13 RIA was reported by NIAMDD.

4.1 Cross-reaction with other hormones and hormone subunits.

In order to provide a reasonable basis for quantitative comparisons in discussing cross-reactions with other hormones or subunits, the amounts of hormones (including LH) and subunits will be expressed in terms of 'pure' substances rather than in terms of the often quite impure RIA standards.

a) Rhesus chorionic gonadotropin (RhCG)

The only hormone, other than rhLH, which reacts strongly in the cynLH : anti-hCG R13 system is rhCG. In this RIA, rhCG's relative potency in terms of the rhLH standard is about 70% of its potency in the mouse leydig cell TPA, again in terms of the rhLH standard. This strong cross-reaction with rhCG is not surprising; it is, in fact, to be expected in an RIA which employs and antiserum against hCG.

b) Rhesus LH beta subunit

It appears that, although rhLH B cross-reacts slightly,and in non-parallel fashion,in the cynLH:anti-hCG R13 RIA,the magnitude of the cross-reaction is very small.

c) Rhesus alpha subunit, prolactin and growth hormone

A monkey pituitary fraction containing rhL, prolactin, and GH in relative proportions of 1:2:25 showed no response in the cyn LH : anti-hCG R13 RIA when tested at doses containing up to 120 ng rhL , 240 ng rhPRL, and 3,000 ng rhGH.

d) Serum LH-like substance (Serum LHS)

The substance in monkey serum which cross-reacts so stongly in the ovine : anti-ovine LH RIA (Peckham *et al.*, 1977) but appears to be devoid of LH biological activity was tested in the cyn LH : anti-hCG R13 RIA. Because of the limited supply of the purified preparation, it was tested only at a single dose of 125 ug, equivalent to 62.5 ng of rhLH in the ovine : anti-ovine RIA. In the cynLH : anti-hCG R13 system, it produced a very small but significant response, equivalent to 0.4 ng or rhLH. This low order of cross-reactivity does not appear to represent a problem.

e) Rhesus FSH

There appears to be no cross-reaction with rhFSH in the cyn LH : anti-hCG R13 system.

f) Rhesus TSH

Gonadotrophin-free sera from three

thyroidectomized monkey showed no response in the cynLH : anti-hCG R13 system. These sera contain from 10 to 50 ng of TSH per ml and were tested at maximum doses of 200 ul, so it appears that as much as 100 ng of TSH produces no response in this RIA. Similarly, a pituitary fraction containing about 25 ng of rh TSH per ml and essentially free of LH, LH B, and FSH failed to elicit a response when tested at 200 ul.

2) Effect of serum

Gn-free monkey serum has no effect on the specific binding of cynLH tracer by anti-hCG R13. In addition, the responses to various quantities of standard run in the presence and absence of Gn-free serum are not significantly different.

Validation of the cyn LH : anti-hCG R13 RIA

Highly purified cynLH, sera from ovariectomized and orchidectomized rhesus monkeys, and rhesus monkey serum taken during the preovulatory surge all showed log dose-response curves which were paralleled to that of the rhesus pituitary LH standard (NICHD-rhLH). Estimates of their LH content by the cynLH : anti-hCG R13 system were consistent with potency estimates based on the mouse leydig cell TPA and the rhLH : anti-hCG # 267 RIA.

Sera from infant, hypophysectomized, and estrogen treated male rhesus monkeys all showed undetectable LH

levels in both RIA system and the TPA. Assay of daily samples from normal, cycling female rhesus monkeys by the cynLH : anti-hCG R13 sytem showed the expected preovulatory surges of LH and correlated highly (r = 0.96) with values obtained by the rhLH : anti-hCG # 267 system. The expected circhoral patterns of LH were found in samples taken at 20 minute intervals from ovariectomized rhesus monkeys, and again there was a high degree of correlation (r = 0.94) between values obtained by the two RIAs. Furthermore, preliminary testing of a number of serum samples from pigtail monkeys (*Macaca nemestrina*) suggests that the cynLH : anti-hCG R13 RIA will prove equally suitable for measuring circulating LH in this species.

VIII Estradiol 17-B (E_2)

Estradiol was determined by RIA as described by Abraham *et al*(1972) and Hubl (1980). In the case of cynomolgus serum E_2, each sample was extracted twice and did not purified before assay. Antiserum provided by WHO Matched Programme is highly specific for E_2.

Reagents and preparation

1. E_2 standard

Standard E_2 in ethanolic solution at a concentration of 160 nmol/l, was divided into 100 ul and stored at 4°C until use. When required, 10 ml of buffer S

(see appendix) was added and warmed to 40°C for 30 mins, cooled to 4°C and mixed vigorously before use. This solution contained 1.6 nmol/l E_2 and was stable for 2-3 weeks when stored at 4° C.

Six serial dilutions (Standard : buffer 1:1) of E_2 within a range of 25-800 fmol/tube were prepared.

2. E_2 tracer

Tritiated estradiol-17B (2,4,6,7-^{3}H-E_2, specific activity 250 uCi) was diluted to obtain 10 uCi/ml with 25 ml of toluene : ethanol 9:1 solution and stored as the stock solution at 4°C. Working tracer solution was prepared from evaporation of 150 ul of the stock solution and re-dissolved in 15 ml assay buffer S. The time necessary for re-dissolvation of tracer was 30 minutes. This solution was sufficient for one assay of 100 tubes and contained 100 nCi/ml. working solution was discarded at the end of the assay.

3. Recovery tracer

Prepare tracer to monitor recovery by taking 100 ul stock tracer solution and re-constituting to 1 ml with buffer S after evaporating the solvent.

4. Antiserum

The antiserum was raised in rabbit against estradiol-17B-3-O-carboxymethyloxene-BSA. It was provided

in lyophilized form and kept at 4°C. Immediately before use, it was re-constituted in 10 ml buffer S to give the final dilution of 1:210,000.

5. Absorbent

Dextran-coated charcoal separation method was employed to absorb the free from bound forms (Herbert *et al.*, 1965). Reagents were prepared (see appendix). Charcoal suspension must be chilled at 4°C and well mixed before use immediately.

5. Counting solution

Prepared reagents were described in the appendix part.

Determination

1. Extraction :

1.1 Extraction of the recovery

The recovery of extraction (86.50 - 89.00%) was stable from assay batch to assay batch.

200 ul of additional samples were aliquoated and then mixed with 20 ul of recovery tracer, left and allowed to equilibrate with serum binding protein for 30-60 minutes at room temperature. If extraction was carried out soon after the addition of E_2 tracer, the recovery of the latter would be greater than that of the plasma E_2 and

the level of plasma E_2 would be underestimated (Abraham *et al.*, 1971). The 'recovery total' tubes were prepared by adding 20 ul of 'recovery tracer' plus 500 ul of buffer S directly to the counting vials. The samples were extracted with 5 ml of fresh diethyl ether by vortex mixing for 1 minute and then left to be settled. After clear separation of the two phases, the low phase (plasma) was quick frozen by dipping in acetone containing chips of dry ice. Ether was then decanted to glass tubes. The extraction was repeated and transfered to a counting vial and evaporated to dryness. These recovery vials were re-dissolved in 500 ul buffers. Scintillation fluid was then added to the recovery total tubes and the 'average' recovery tubes. They were counted in beta counter for 5 minutes and calculated the percentage recovery for each sample by divided the counts obtained in average recovery tubes by the 'recovery total' tube counts. Additionally, at least two tubes for 'ether blank' were prepared.

1.2 Extraction of the unknown serum and quality control

Before assay, the unknown serum and quality control must be extracted in order to isolate the steroids from the interfering substances present in the biological fluid. The volume of 200-500 ul monkey serum was extracted with approximately 10 volumes of ether (Abraham *et al.*, 1971).

E_2 assay protocol

Day 1 : 200 ul of each sample of the recovery, unknown, quality control was extracted that earlier described and dissolved in buffer S (500 ul). Six serial dilutions of E_2 standard were prepared. Excluding the NSB tubes, 100 ul of antiserum was added to all other tubes. Then E_2 tracer was added to each tube and mixed well, covered, incubated at $4^{o}C$ for 18-24 hr (overnight).

Day 2 : In ice bath, 200 ul of continuously mixed dextran coated charcoal suspension was rapidly added to all tubes except Tc tubes. All tubes were mixed immediately by shaking the rack and incubated for 20 minutes in an ice bath, centrifuged at 1500 g, at $4^{o}C$ for 15 minutes. The supernatant in each tube was decanted into a counting vial, added with 5 ml of scintillation fluid, capped and shaked well. All vials were left for at least 1 hr and then counted for 5 minutes in a beta counter.

Validation of E_2

1. Sensitivity

The sensitivity of this E_2 assay was 21 fmol/tube.

2. Precision

The coefficient variation of within-assay was 6.08% for the low quality control, 6.98% for the

intermediate quality control and 7.91% for the high quality control. For the between assay variability, % coefficient variations of low, intermediate and high quality controls were 6.13%, 10.33% and 10.89% respectively. The working range was 24.79 - 750 fmol/tube at the % CV 13.94 - 10.45%.

3. Accuracy

The correlation coefficient between the determined and added values was 0.98.

4. Specificity

The cross reaction of antiserum with steroids likely to be presented in the serum were tested as the following table (WHO Matched Reagent Program, 1986).

Cross-reaction substance	% Cross-reaction
Cortisol	0.001
Testosterone	0.0002
Estrone	1.700

IX Progesterone

Progesterone reagent were also provided by WHO Matched Programme (1986).

Reagents and preparation

1. Progesterone standard

The standard is provided in ethanolic solution at a concentration of 250 nmol/l in an ampoule. After opened the ampoule, 3 x 100 aliquoats were transfered into the screw capped bottles and stored at $4^{o}C$. When required, 10 ml of buffer S was added and mixed vigorously . The solution contained 2.5 nmol/l. Six serial dilutions of progesterone within the range of 39-1250 fmol/500 ul (tube) were prepared shortly before assayed.

2. Progesterone tracer

Tritiated progesterone (1,2,6,7 ^{3}H-Progesterone, 100 uCi) was diluted to obtain 10 uCi/ml with 10 ml of high purity tuluene. Working tracer solution was prepared as followed, 150 ul of the stock solution was taken into a tube and evaporated the solvent and re-dissolved in 15 ml assay buffer S, for at least 30 minutes. This working solution contained 100 nCi/ml (3.7 KBq/ml).

3. Recovery tracer

The recovery tracer for monitoring recovery was prepared by taking 100 ul stock solution and re-constituting with 1 ml of buffer S after evaporating the solvent.

4. Anti-progesterone serum

Anti-progesterone is provided in lyophilized form. The contents of antiserum was reconstituted with 10 ml of assay buffer S and mixed well. The mixture should be stood for 5-10 minutes and mixed before used.

5. Absorbent

See appendix

6. Counting Solution

See appendix

Progesterone Assay protocol

Day 1 : Duplicate samples were aliquoated in quantities of 50 ul for the luteal phase and of 100 ul for the follicular phase. 100 ul of each the recovery samples, quality controls, and unknowns was extracted for one time. The remainder steps were similar to the method of E_2 extraction. The recovery of the steroid extraction was between 88-93%. Dried samples were dissolved in buffer S 500 ul and mixed well. The serie of progesterone standards were aliquoated (500 ul). Excluding the Tc and NSB tubes, the first antibody was added in the amount of 100 ul. Progesterone tracer (100 ul) was added to all tubes , mixed and incubated at $4^{o}C$ for 18-24 hours.

Day 2 : Aliquoats (200 ul) of chilled charcoal

suspension in ice bath (4°C) was added rapidly into all tube except Tc tubes. They were mixed by shaking the rack and then incubated for 15 minutes. Consequently, all tubes (except Tc tubes) were centrifuged at 1500 g,4°C for 15 minutes. The supernatants were decanted into counting vials and added 5 ml of scintillation fluid. They were counted for 5 minutes in a beta counter.

Validation of Progesterone

1. Sensitivity

The sensitivity of progesterone assay was 35 fmol/tube.

2. Precision

Coefficient variations of low,intermediate and high levels of quality control in the within-assay were 5.12%, 4.50% and 9.55%, respectively. In the between-assay, 16.02%, 9.75% and 7.75% of coefficient variations for low, intermediate and high quality controls were respectively exhibited. The working range was 67.95 - 1250 fmol/tube at % CV 12.76 - 5.37%.

3. Accuracy

The correlation coefficient between the determined and added values was 0.96.

4. Specificity

The cross-reactions of some substances mostly presented in blood were tested by WHO Matched Reagent Programme (1986) and listed below.

Cross reacting substances	% Cross-reaction
Cortisol	< 0.01 %
Testosterone	< 0.30%
17-hydroxyprogesterone	< 3.00%
20 -dihydroxyprogesterone	< 3.00%

Statistical Analysis

The results were expressed as mean ± S.D. The mean differences between before and after experiment study were tested by paired t'test. Spearman's rank correlation coefficiency was used in the statistical evaluation of correlation between two parameters. A probability of $P < 0.01$ and $P < 0.05$ were considered to be statistically different.

Calculations

The unknown , quality controls, % CV, the response error of counts and error analysis of these hormone assays were calculated and analysed by using the WHO Immunoassay Programme (Version PC. 5.1) as described by Edward, 1987.