CHAPTER 2

MATERIALS AND METHODS

A. MATERIALS

animals

Ten sexually mature female Macaca fascicularis with body weights ranging from 3.5 - 4.5 kgs. were used in this study. Five monkeys in this group showed evidence of galactorrhea, some displayed irregular menstrual cycles and all had problems of reproductive failure. The remaining were selected as a control group. These monkeys had regular menstrual cycles of 28 - 32 days, attained good breeding records and ahd normal mating behavior.

The monkeys come from the same colony at the Primate Center of Chulalongkorn University which houses up to 120 monkeys of both sexes and various ages. All were kept in individual squeeze cages in quarters with natural light and good ventilation. Additional fluorescent light was provided from 06.00 - 18.00 hrs.

Monkeys were fed daily in the morning with monkey chow (Pokphand animal Feed Co. Ltd. Thailand) and in the afternoon with fresh cucumbers, pineapples, bananas and sweet potatoes as supplements.

The animals were housed under this condition and kept on this ration for more than 2 years before this study was implemented.

Clinical Observations

Five monkeys which showed galactorrhea were as follows :

Monkey # 24 : is about 12 years old, she is a small monkey but slightly over-weight and inert. She weighed 3.8 kg.. This monkey has never been pregnant but displays normal menstrual cycle and mating behaviour. Though keenly receptive during mating, she still fails to concieve. Sexual skin is pale and rather inconspicuous.

Monkey # 11 : is over 15 years old and is one of the smaller monkeys with a timid disposition. She weighed 2.9 kg. She has a history of obortion due to mishandling during procurement. Menstrual cycle is normal but has periods of menstrual absence and recerrence. Sexual skin is obscure.

Monkey # 74 : is about 10 years old and weighed 3.2 kg, slightly underweight. She is easily excited and constantly shows signs of fear. Mating behaviour is normal and receptive but she has a very low conception rate. Sexual skin is noticable and she has normal and constant menstrual cycle. She got the delivery phenomenon for two time; once in 1980 and again in 1981.

Monkey # 58 : is about 10 years old and appears to be a very healthy monkey. She weighed 4.2 kg. In mating, she is receptive and displays an easily noticed sexual skin. Her menstrual cycle is not quite constant with periods of menopause and recurrence. She also had a baby in 1980.

Monkey # 29 : is over 15 years old. She is a small monkey, 2.9 kg. body weight; with a timid and easily frightened disposition. Menstrual cycling is normal and there is a history of delivery in 1975. In mating, this monkey is not receptive because of fear of the mate. Her sexual skin is inconspicuous.

All these monkeys were acquired and not bred in the laboratory These monkeys exhitited symptoms of galactorrhea at least 3 months before study, with a special note on # 74 which secreted immence amounts. All of them are failed to get pregnancies for a long time. They had been mated many times before this study, and every mid-cycle while this study was being conducted.

Chemical

BSA (Bovine serum albumin)	Sigma Chemical Company, USA
Charcoal Reagent	WHO RIA Reagent Program
Dextran	WHO RIA Reagent Program
Diethyl Ether	E. Merck; Germany
Disodium hydrogen phosphate	E. Merck; Germany
EDTA	J.T. Baker Chemical Company Co., U.S.A.
Ethanol (absolute)	E. Merck, Germany
Gelatin	Difco Laboratories, U.S.A.
Hydrochloric Acid	BDH Chemicals Ltd., England
PPO (2, 5 Diphenyloxazole)	Sigma Chemical Company, U.S.A.
Sodium Azide	Sigma Chemical Company, U.S.A.
Sodium Chloride	BDH Chemicals Ltd., England
Sodium Hydroxide	BDH Chemicals Ltd., England
Sodium Dihydrogen Phosphate	E. Merck, Germany
Toluene	E. Merck, Germany
Triton X-100	Sigma Chemical Company, U.S.A.
Thiomersal	Sigma Chemical Company, U.S.A.

All chemicals were obtained commercially and were of reagent grade or better. Charcoal and Dextran were supplied from WHO RIA Matched Reagent Program as in the steroid RIA kits.

Hormone and Antisera

Human Estradiol, Progesterone, Prolactin and monkey Luteinzing Hormone were obtained from WHO RIA Matched Reagent Program. Normal Rabbit Serum (NRS) was also supplied from this program.

Tracers

(2, 4, 6, 7, 16, 17, ^{3}H) - Estradiol and (1, 2, 6, 7, ^{3}H) - Progesterone were purchased from Amersham International Limited.

(^{125}I)-LH and (^{125}I)-Prolactin were obtained from WHO RIA Matched Reagent Program.

B. METHODS

Experimental Procedures

1. Baseline data of serum prolactin, estradiol Progesterone and luteinizing hormone in normal cycling control group.

Five sexually mature female monkeys, aging of 5 - 8 years old and had regular menstrual cycles of 28 - 32 days with good breeding records were selected as a control group.

Blood samples were obtained from the first day of menstrual cycle until the end of that cycle in each monkey. All hormone levels were evaluated to form the baseline data as the control group.

2. Baseline data of serum prolactin, estradiol progesterone and luteinizing hormone in galactorrhea monkeys.

Blood samples were obtained from the first day of menstrual cycle until the end of the cycle in the regular group and until the 100^{th} day in the irregular group. These serum hormone levels were also determined and used to create the baseline data of galactorrhea group as well.

3. Effect of bromocriptine on serum prolactin, estradiol progesterone and luteinizing hormone in galactorrhea monkeys.

Bromocriptine (PARLODEL 2.5 mg; Sandoz Ltd. Basle . Switzerland) was given orally by force feeding to all galactorrhea monkeys in two daily divided doses (8.00 a.m. and 17.00 p.m.) for as long as 30 days as shown in figure 3 - 7.

Blood sample were collected one day before Bromocriptine treatment as a pretreatment sample and every other day throughout the 30 days of treatment and also during the 12 day post-therepeutic peroid. The first sample during treatment was taken 2 hours after the administration of the first dose.

The serum hormone levels were carefully determined to observe an inclination or fluctuations in relationship to the therapeutic program.

4. Effect of bromocryptine on fertility improvement in galactorrhea monkeys.

Bromocryptine was given orally by forve feeding to galactorrhea monkeys in the in 4 monkeys, monkey # 74, monkey # 29, monkey # 11 and monkey # 58 same dose (2.5 mg in two daily divided

doses) but long for 3 months. During every mid-cycle of these monkeys, they have mating periods with healthy males as shown in figure 3 - 7. The abdominal pulpation (after 3rd week) or urinary detection by HAI test during day 17 - 27 of post-mating (Varavudhi et al., 1982) were performed to detect pregnancies.

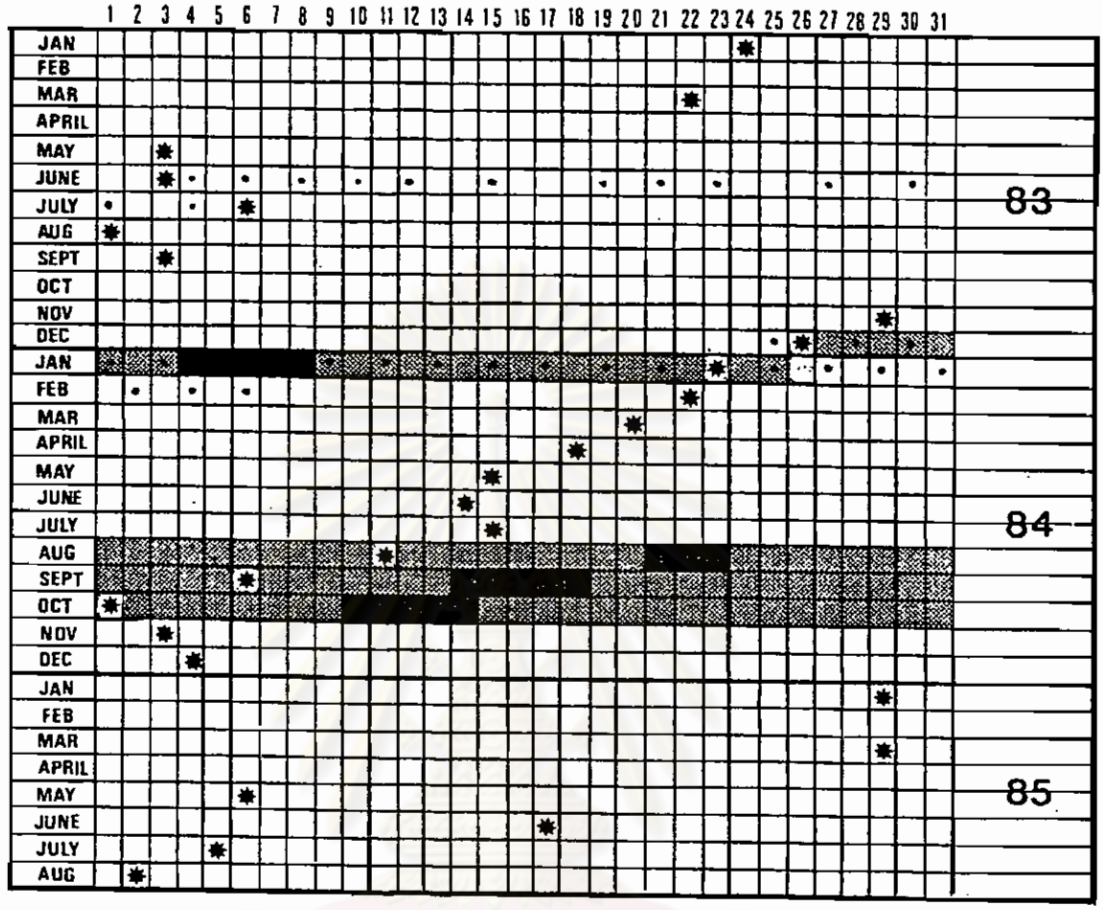

FIGURE 3

first day of menstrual cycle

The day of blood collections

The day of bromocryptine treatment

The day of mating during bromocryptine treatment

Fig. 3 Menstrual records of the monkey # 74 and studying protocol

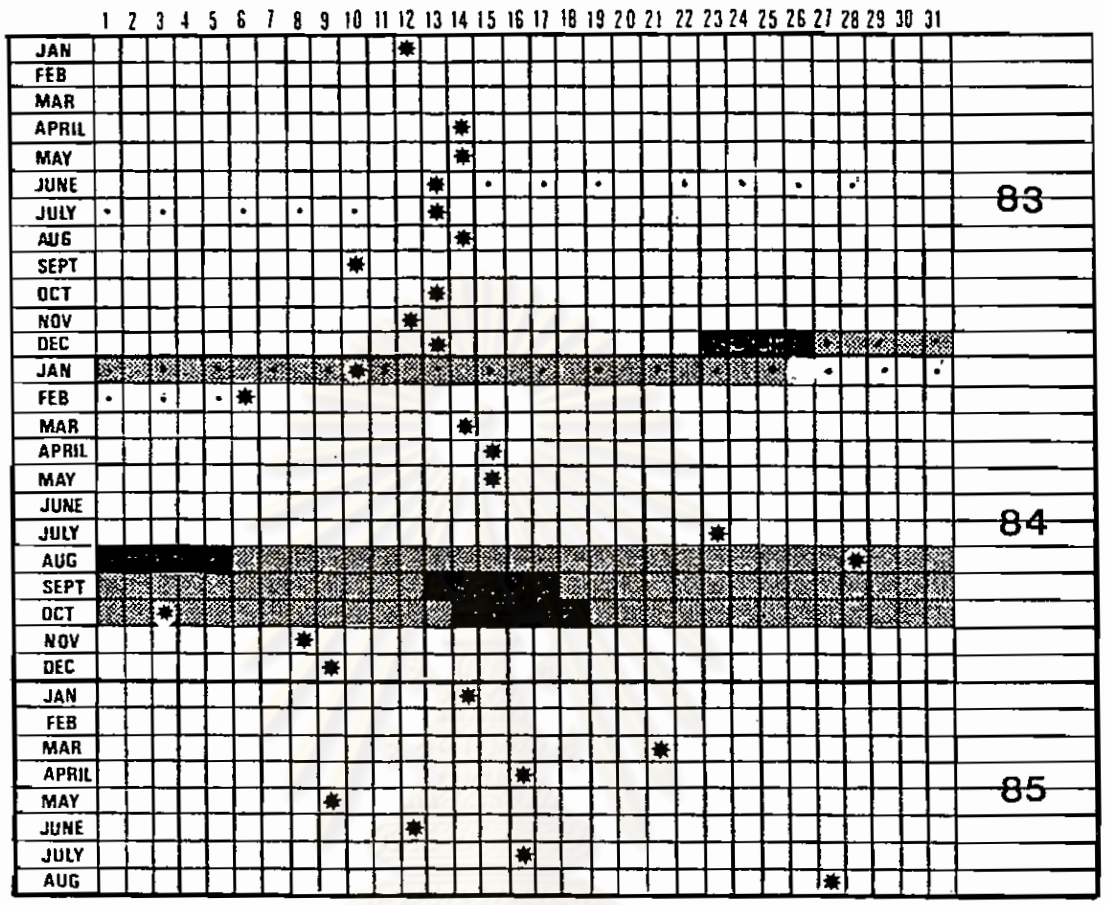

FIGURE 4

first day of menstrual cycle

The day of blood collections

The day of bromocryptine treatment

The day of mating druing bromocryptine treatment

Fig. 4 Menstrual records of the monkey # 29 and studying protocol

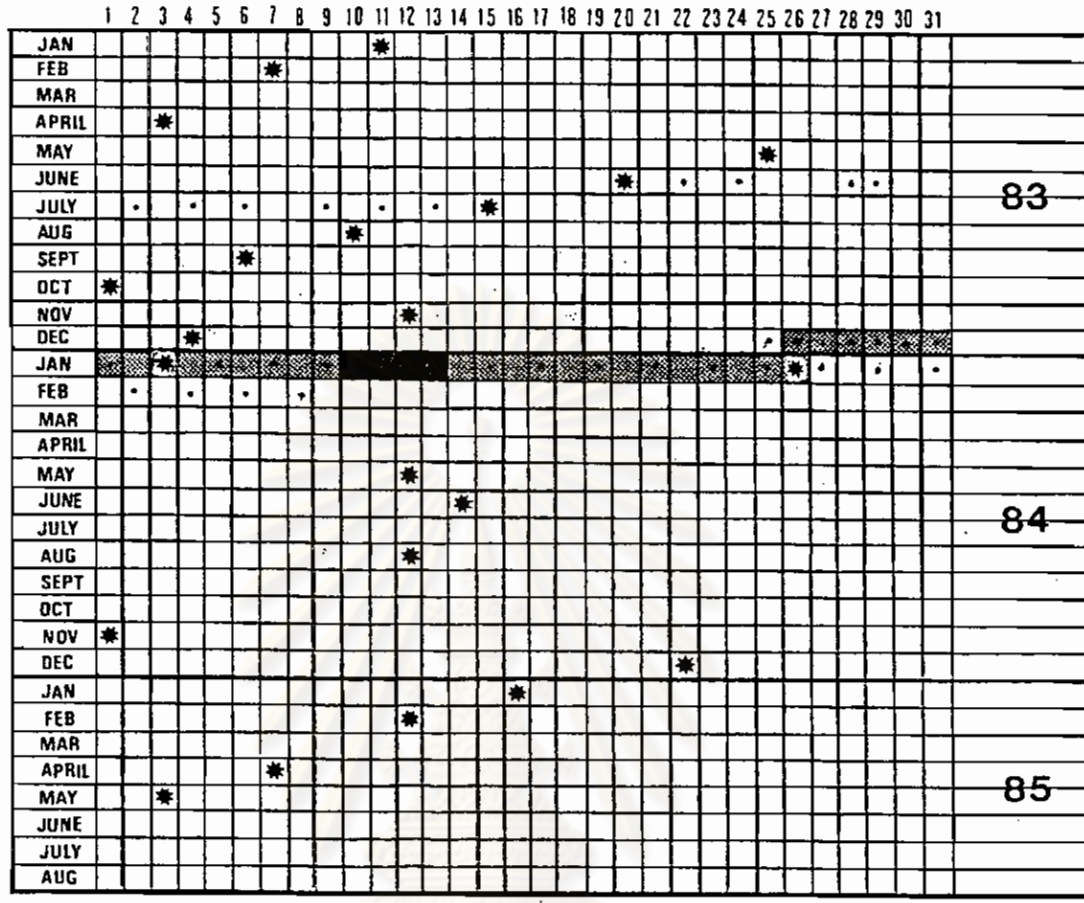

FIGURE 5

✸ first day of menstrual cycle

● The day of blood collections

▒ The day of bromocryptine treatment

▓ The day of mating during bromocryptine treatment

Fig. 5 Menstrual records of the monkey # 24 and studying protocol

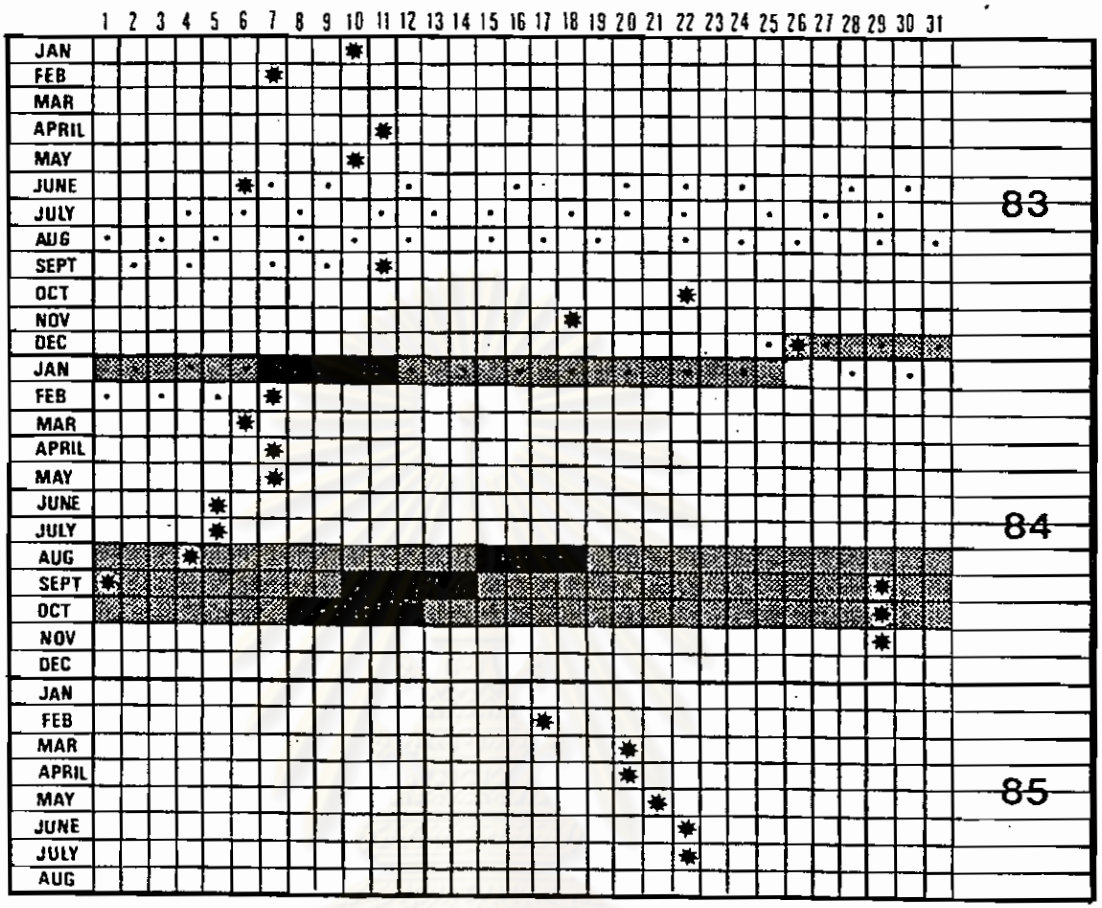

FIGURE 6

first day of menstrual cycle

The day of blood collections

The day of bromocryptine treatment

The day of mating during bromocryptine treatment

Fig. 6 Menstrual records of the monkey # 11 and studying protocol

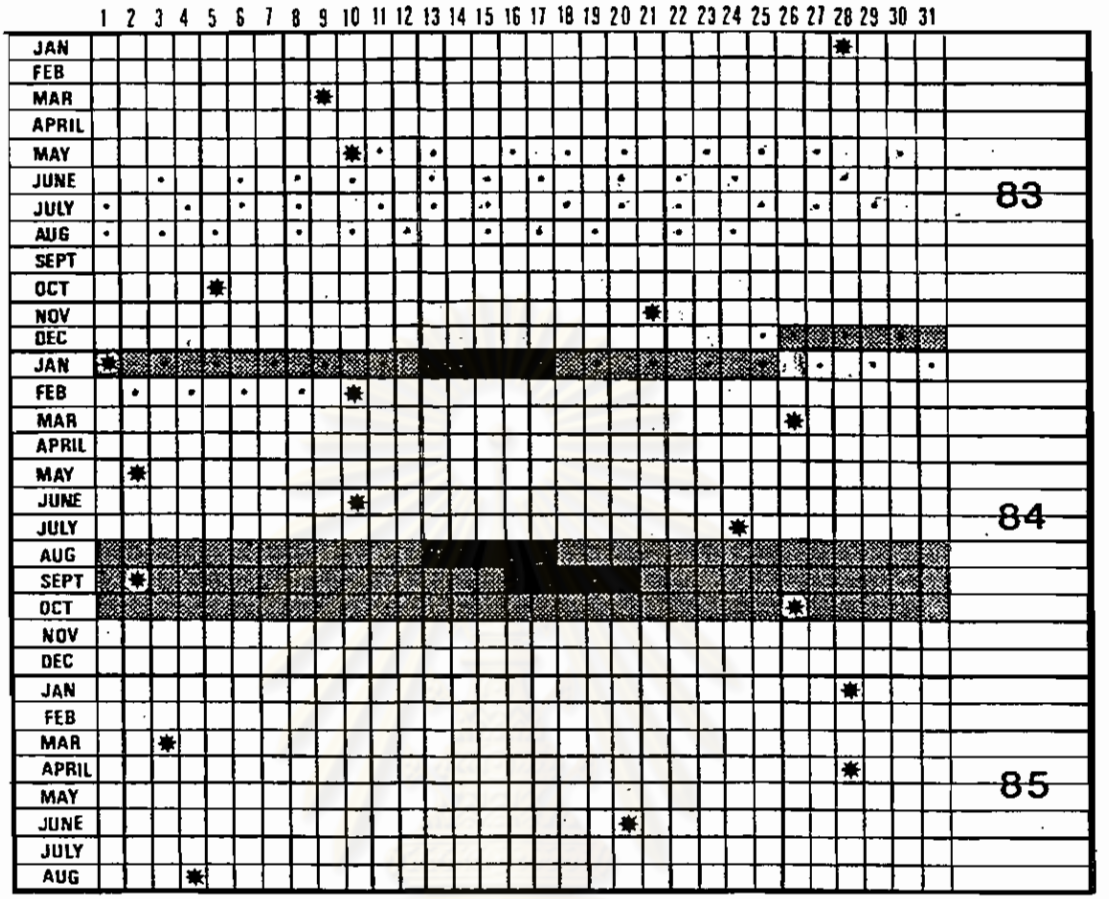

FIGURE 7

first day of menstrual cycle

The day of blood collections

The day of bromocryptine treatment

The day of mating during bromocryptine treatment

Fig. 7 Menstrual records of the monkey # 58 and studying protocol

Blood Samples

Blood samples of 3 ml. were obtained at 09.30 - 11.00 a.m. through femoral venepuncture in non-anaesthesized animals. These samples were allowed to clot and stored overnight in the refridgerator. Serum was then seperated by centrifugation at 4°C. and stored at -20°C. until assayed. All hormones were analyzed and determined from the same samples.

The sampling was done every other day commencing on the first day of menstruation in the initial cycle up to the first menstruating day of the following one, thus, completing a full menstrual cycle of about 28 - 32 days.

As for the animals with irregular menstrual cycles or displayed amennorrhea states druing the sampling (monkey # 11 and # 58), blood collection continued also on an every other basis for a period of 100 days.

To prevent anemic states; all animals used in this study were given oral hematinics (NUTROPLEX LIQUID^{R} : United American Pharmaceutical Inc., U.S.A.) throughout the conduct.

1. Progesterone Radioimmunoessay.

Reagents :

1. Assay Buffer (Buffer S; BS)

This buffer is used as a diluent for all the reagents in steroid assays.

Prepared by dissolving 1.0 gm. gelatin in 300 ml. warm distilled water first; then add the substances as described below;

3.10 g. Sodium Dihydrogenphosphate-1-hydrate

(NaH_2PO_4; m.w. 120)

11.6 g. Disodium Hydrogen-hosphate (anh.)

(Na_2HPO_4; m.w. 142)

8.8 g. Sodium Chloride

0.1 g. Thiomersal (Merthiolate)

then use distilled water to add up the solution to 1 litre. Let the solution be mixed throughly. Adjust the pH to range between 7.2 - 7.4. This solution can be stored at $4^{o}C$. for up to 1 month.

2. Standards

Progesterone standards bottled in concentrations of 24 nmoles/L; serves to be the stock solution.

Six steroid serial dilutions (standard : buffer = 1:1) of Progesterone within the range of 37.5 - 1200 fmol were prepared shortly before usage from a stock solution stored at $4^{o}C$.

The table below summarizes the procedure described.

Standard	Transferring Solution		Buffer S (ml)	Concentration (fmol/tube)
	Solution	Amount (ml)		
Std. 1	Stock Std.	0.5	4.5	1200
Std. 2	Std. 1	2.0	2.0	600
Std. 3	Std. 2	2.0	2.0	300
Std. 4	Std. 3	2.0	2.0	150
Std. 5	Std. 4	2.0	2.0	75
Std. 6	Std. 5	2.0	2.0	37.5

3. Tracer

1, 2, 6, 7 ^{3}H-Progesterone (250 uCi) was diluted up to 25 ml analytical grade of Benzene : Ethanol; 9 : 1 and stored as a stock solution of 10 uCi/ml at 4^{o}C.

Shortly before usage; the working tracer was prepared by evaporating the stock and diluted 1 : 100 in buffer S; to obtain 100 uCi per ml. in assay

4. Antiserum

The antiserum was raised in intact rabbit against progesterone-3-O-carboxymethyloxene-BSA and bottled in lyophilised form. Immediately before use, the contents should be reconstituted with 10 ml of Buffer S and will give the final dilution as 1 : 70,000 in the assay tube.

5. Absorbant

0.625 g. activated charcoal and 0.0625 g. dextran were suspended in 100 ml assay buffer. This charcoal suspension can be

stored at 4°C. for 1 month and needs to be thoroughly mixed before and during usage.

6. Counting Solution

3 litres of scintillation fluid can be prepared by dissolving 15 g. PPO (2, 5 diphenyloxazole) in 2 litres of Toluene, then added with 1 litre of Triton X-100, mixed and stored in brown bottles.

Protocol

Day 1 Duplicate samples were aliquoted in quantities of 50 ul for the luteal phase serum and 125 ul for the follicular phase serum. Background, quality control samples and recovery samples were included in every assay.

Samples were extracted by adding 5 ml of fresh anhydrous diethylether and vortexed simultaneously for approximately 1 minute. The aqeuous phase was allowed to become frozen in a dry ice/ethanol bath, and the organic phase of ether supernatant could be decanted. This ether fraction was evaporated by using triblock heater at 70°C. and redissolved in 500 ul assay buffer. With an exception of the NSB tubes, 100 ul of antiserum was added to the samples, Standards, and Bo tubes. Then 100 ul of tracer was added to each tube, vortex mixed; covered and incubated overnight at 4°C.

Day 2 In an ice bath, 200 ul of charcoal-dextran suspension which was continuously cool mixed on ice by magnetic stirrer was rapidly added to all tubes except for the Tc tubes (total counts). This must be done in order to prevent the incubation time from differing too much between each assay tube. 15 minutes after the last tube was vortexed; all tubes

were centrifuged for 15 minutes at 2500 rpm, $4^{o}C$.

After centrifugation the supernatant containing the antibody-bound fraction of each tube was rapidly decanted into a scintillation vial. Then 5 ml of scintillator was added, the vials were capped, shaked; labelled; left for at least 1 hour and counted for 5 minutes per tube in the Beta counter.

Validation and Quality Control

Specificity the cross reaction of this antiserum with steroids likely to be present in the serum were tested as in the following table (WHO Matched Reagent Program 1981). The cross reaction was calculated at B/Bo of 50 %

Cross Reacting Substance	% Cross Reaction
Cortisol	< 0.01 %
Testosterone	< 0.3 %
17∝-hydroxyprogesterone	< 3.0 %
20∝-dihydroprogesterone	< 3.0 %

Sensitivity The sensitivity of this assay was 20 fmol/tube

Precision for intra-assay variability, %cv in a cynomolgous serum pool for 8 replicants was 5.4 %

For inter-assay variability, % cv for 6 assays was 12.3 %

Binding binding for 6 assays was 45 - 50 %

Accuracy The accuracy for the assays was 80 - 85 %

2. Estradiol Radioimmunoassay

In this study we employed the RIA technic in accordance with the WHO Method Manual, Special Program of Research; Development and Research Training in Human Reproduction 1981.

Reagents :

1. Assay Buffer

Buffer S as described beforehand was used.

2. Standards

Estradiol standards were provided as a stock solution at the concentration of 16 nmol/L.

Six serial dilutions (Standard : Buffer S, 1 : 1) of estradiol within a range of 25 - 800 fmol was prepared shortly before use from a stock solution stored at $4^{\circ}C$.

The procedure is shown in a summarized table as follows.

Standard	Transforming Solution		Buffer S (ml)	Concentration (fmol/tube)
	Solution	Amount (ml)		
Std. 1	Stock sol.	0.5	4.5	800
Std. 2	Std. 1	2.0	2.0	400
Std. 3	Std. 2	2.0	2.0	200
Std. 4	Std. 3	2.0	2.0	100
Std. 5	Std. 4	2.0	2.0	50
Std. 6	Std. 5	2.0	2.0	25

3. Tracer

2, 4, 6, 7, 16, 17 - ^{3}H Estradiol (250 uCi) was diluted up to 25 ml with analytical grade of Benzene : Ethanol, 9 : 1 and stored as the stock solution at 4°C. This stock solution contains 10 uCi/ml.

Shortly before use; the working tracer was prepared by evaporated the stock and diluted 1 : 100 with Buffer S. to contain 100 uCi/ml in the assay.

4. Antiserum

The antiserum was raised in intact rabbit against Estradiol -3-0-carboxymethyloxene-BSA. She was bottled in lyophilised form and could be stored at 4°C. Immediately before use, she was reconstituted in 10 ml Buffer S giving the final dilution in the assay tube as 1 : 56,000.

5. Absorbant

Charcoal suspension as described beforehand was used.

6. Counting Solution

Prepared as described beforehand.

Assay Procedure

The assay contained a duplicate of each unknown sample, triplicate of standards (Std), maximal binding (Bo), quality control samples (Qc), Recovery samples (R), Total count (Tc), non-specific binding (NSB), solvent blank and background; all as in the Progesterone assay.

Protocol

Day 1 Each sample was aliquated of 200 ul including the background; Quality comtrol samples and Recovery samples.

Samples were extracted by fresh anhydrous diethylether as previously described in the Progesterone determination procedures. The organic phase of ether was decanted after frozen in a dry ice, ethanol bath and evaporated to dryness at 70°C. by a triblock heater and re-dissolved in 500 ul Buffer S.

Excluding the NSB tube; 100 ul of antiserum was added to all tubes including a set of standard tubes. The tracer was later added to each tube which were vortexed, covered and incubated overnight at 4°C.

Day 2 On ice; 200 ul of continuously mixed charcoal-dextran suspension was rapidly added to all tubes except for the Tc tubes. 15 minutes after the last tube was vortexed, all were centrifuged for 15 minutes at 2500 rpm, 4°C.

The supernatant in each tube was decanted into a counting vial, added with 5 ml of Scintillation, capped, shaked, left for at least 1 hour and counted for 5 minutes each in the Beta counter.

Validation and Quality Control.

Specificity The corss reactions of antiserum with steroids likely to be present in the serum were tested as in the following table (WHO Matched Reagent Program, 1981). The cross reaction was calculated at B/Bo of 50 %.

Cross-reaction Substances	% Cross reaction
Cortisol	0.001
Testosterone	0.0002
Estrone	1.7

Sensitivity The sensitivity of this assay was 17 fmol/tube

Precision For intra-assay variability, % cv in a cynomolgous serum pool for 8 replecants was 7.6 %

For inter-assay variability; % cv for 6 assays was 13.2 %

Binding % binding for 6 assays was 45 - 50 %

Accuracy The accuracy for the assays was 85 - 90 %

3. Prolactin Radioimmunoassay

In this study, serum prolactin was measured through RIA methods in accordance with WHO Matched Reagent Program, 1981 using heterologous assay of human kits which seperates bound hormone from the free form by the second antibody method.

Reagents :

1. Assay Buffer (Buffer P, BP)

This is the peptide assay buffer (P) used as a diluent for all reagents in this assay except for the tracer. It can be

stable for as long as 1 month at $4^{o}C$.

To prepare; dissolve the reagents listed below in distilled or deionezed water.

3.10 g. Sodium dihydrogenphosphate-1-hydrate

11.6 g. Disodium hydrogenphosphate (anh)

8.8 g. Sodium chloride (NaCl)

0.19 g. Thiomersol

5.0 g. Bovine serum albumin (BSA)

EDTA should also be added as a final constituent at an amount of 0.025 M per litre of the prepared buffer. The pH must be adjusted to range between 7.2 - 7.4 by conc. NaOH or HCl.

2. Tracer diluent

The buffer P above puls 0.5 % normal rabbit serum (NRS) serves as a tracer diluent. The NRS is essential for the double antibody seperation stage with the high IgG content. NRS is provided as one of the WHO matched assay reagents.

3. Standards

The prolactin standard contains 2.5 mU of prolactin per vial. The vials had been filled with freeze dried aliquotes of prolactin from a solution of the IRP (International Reference Preperations) for prolactin (code 75/504).

Immediately before an assay, an aliqupte of prolactin standard was reconstituted with 1 ml of Buffer P resulting in a concentration of 2500 mIU/L (Milli-international unit/litre). Six serial dilutions (standard : buffer, 1 : 1) of prolactin standard within a range of 78 - 2500 mIU/L are prepared as summarized in the table.

Standard	Transforming solution		Buffer P (ML)	Concentration (mIU/L)
	Solution	Amount (ml)		
Std. 1	a freeze dried aliquote of Prolactin standard in vial		1	2500
Std. 2	Std. 1	0.5	0.5	1250
Std. 3	Std. 2	0.5	0.5	625
Std. 4	Std. 3	0.5	0.5	313
Std. 5	Std. 4	0.5	0.5	156
Std. 6	Std. 5	0.5	0.5	78

4. Tracer

^{125}I - prolactin had been sent lyophilized in bottles, were redissolved in 1 ml of Buffer P and stored at $4^{o}C$ as the stock solution. For each assay 0.2 ml of this was diluted to 10.5 ml with the tracer diluent.

5. Antiserum

Anti-Human pituitary prolactin store freeze dried at $4^{o}C$ was reconstituted with 10 ml of Buffer P before usage, giving a final dilution of 1 : 400,000 in the assay tube.

This antisera was obtained by innoculating Human Prolactin into intact rabbits and has the efficacy to cross react with Prolactin in cynomolgus monkeys.

6. Second Antibody

The second antibody was provided and stored at $4^{o}C$. Immediately before use; it was diluted with Buffer P in a ratio of 1 : 40

Protocol

Day 1 100 ul aliquotes of duplicate samples of serum; tripicates of standards as well as the Bo tubes were added with 100 ul of antiserum, thus, excluding the Tc and NSB tubes. Then 100 ul of tracer along with 400 ul of Buffer P was added to all tubes which were then vortex mixed, covered and incubated for 48 hrs. at $4^{o}C$.

Day 3 100 ul of the Second antibody which was diluted to 1 : 40 was added to all tubes except for the Tc tubes. The tubes were vortexed, covered and incubated at $4^{o}C$ overnight.

Day 4 The tubes were centrifuged for 45 minutes at 3,000 rpm at $4^{o}C$ then the supernatant was decanted. Subsequently, the precipitate (bound fraction fraction) and the Tc tubes were counted in a gamma-counter for 2 minutes.

Validation and Quality Control

Specificity The WHO IRP 75/504 pituitary prolactin standard has cross reactions also with Human growth hormone in percentages of 0.05. This was calculated at 50 % B/Bo (WHO Matched Reagent Program, 1981).

As with any heterozygous assay for a protein hormone, values measured in this assay cannot be interpreted as absolute values of the hormone (Bangham & Booth, 1972). Therefore, the results were regarded only as relative values of the hormone.

Sensitivity The sensitivity of this assay was 104.12 mIU/L.

Precision The quality control, as the intra-assay variability, % C.V. was 0.64 %, as the inter-assay variability, % C.V. was 4.77 %

Binding % binding was 28.68 %

Accuracy The accuracy for these assays was 96 - 109 %

4. Luteinizing Hormone Radioimmunoassay

In this study, serum LH was measured through a new heterologous RIA system developed for monkey LH by WHO Collaborating Center as described in WHO Method Manual 1984 for Radioimmunoassay of Rhesus Luteinizing Hormone (rhlh) and Rhesus Follicle-Stimulating Hormone (rhFSH).

This system utilizes a highly purified cynomolgus LH as tracer, an anti-HCG serum; and a highly purified rhesus LH as standard.

Reagents :

1. Buffers;

Stock Solution A Dissolve 27.8 g of 0.2 M Sodium dihydrogenphosphate-1-hydrate in 1000 ml distilled water.

Stock Solution B Dissolve 28.39 g of 0.2 M Disodium hydrogenphosphate in 1000 ml distilled water.

1.1 0.1 M Phosphate Buffer pH 8.0

Add 2.0 g of Sodium azide into the mixture of 53 ml Stock Solution A and

947 ml Stock Solution B, then bring up to 2000 ml by distilled water.

1.2 Tracer Buffer (1 % BSA-Phosphate Buffer pH 8.0)

Dissolve 10.0 g BSA in 1000 ml of the Phosphate Buffer in 1.1; this buffer is used for the dilution of Tracers.

1.3 Sample Buffer

7.2 g Sodium chloride and 1.0 g Sodium azide are added into 100 ml Tracer Buffer, and then diluted to a total of 1000 ml with distilled water. This buffer is also used as the diluent of sample and standard.

1.4 Second Antibody Buffer (phosphate - EDTA Buffer)

Dissolve 18.6 g disodium ethylendiamine tetraacetic acid (EDTA) in 800 ml of the Phosphate Buffer in 1.1 and adjust the pH with 5N NaOH to bring it to pH 8.0; then make the volume up to 1000 ml with Phosphate Buffer in 1.1

1.5 First Antibody Buffer

Add 50 ul of Normal Rabbit Serum (NRS) into 100 ml Tracer Buffer. This can be used for the dilution of first antibody.

2. Standards;

Rhesus LH standard (WP-XV-rhLH-RPi) is provided in lyophilized from which contains 1.0 ug of this lyophilized material in each vial. Immediatly before use, dissolve the contents of the vial in 2.5 ml Sample Buffer and this is designated as 'Dilution A' which resulting in the highest dose (80 ng/200 ul). Then, the further six serial dilutions within a range of 1.25 - 40 ng/200 ul are made from this by the following procedure.

Standard	Transformaing Solution		Standard Buffer (ml)	Concentration (ng/200 ul)
	Solution	Amount (ml)		
Std. 1	Lyophilized form of LH standard in vial		2.5	80
Std. 2	Std. 1	1.0	1.0	40
Std. 3	Std. 2	1.0	1.0	20
Std. 4	Std. 3	1.0	1.0	10
Std. 5	Std. 4	1.0	1.0	5
Std. 6	Std. 5	1.0	1.0	2.5
Std. 7	Std. 6	1.0	1.0	1.25

3. Tracer

A highly purified cynomolgus LH (WP-XV-63) was iodinated to be used as tracer and being provided in lyophilized form. The contents should be dissolved in 2.0 ml of Tracer Buffer and stored at $4^{\circ}C$. as the stock. For each assay; an aliquote of this solution should be further diluted with the same buffer to obtain approxmately 20,000 disintegration per minute in 100 ul.

4. Antiserum

Antiserum to hCG is diluted 1 : 100 with buffer and aliquote of 1.0 ml is lyophilized in each vial. Before usage; this lyophilized material should be carefully reconstituted with 10 ml of First Antibody Buffer as the working dilution and gave the final dilution of 1 : 40,000 in each assay tube.

5. Second Antibody

Donkey serum containing anti rabbit gamma globulins is provided undiluted in 1.0 ml aliquotes and stored at -20°C. Immediately before use, it was diluted with Second Antibody Buffer in the ratio of 1 : 30.

6. Low Gonadotrophin Serum (LGS)

When assaying, it is necessary to add LGS to the standard tubes to compensate for the effect of serum proteins on the second antibody precipitation reaction. This extremely low gonnadotropin content can be obtained from hypophysectomized and estradiol treated monkeys, but in this study, we use pooled monkey serum absorbed with First Antibody (1 : 100) by mixing 10 ml pool serum in 1 vial of antibody (lypphilized form of dilution 1 : 100).

Protocol

Day 1 100 ul of tracer and first antibody are added to 200 ul of duplicate standard and 100 ul of duplicate sample in proportion indicated in table. The tubes are then vortexed mixed and incubated at 4°C for 72 hours.

Day 4 Second antibody and LGS are added to the tubes as shown in the table. Mix and incubate at $4^{\circ}C$ for 24 hours as the second incubation.

Day 5 Add 3.0 ml of cold distilled water to all tubes. The tubes then centrifuged at 3200 rpm, $4^{\circ}C$ for 45 minutes, Unbound fraction is then carefully removed by carefully decanting the supernatants. The contents of these tubes airdried by inversion on a pad of absorbant tissue. The precipitate (bound form) is then counted in a gamma counter for 2 minutes.

Immunoassay Procedure of Rhesus Luteinizing Hormone (rhLH)

	DAY 1				INCUBATE	DAY 4			INCUBATE	DAY 5
	Sample (Std)	Sample Buffer	Tracer	Antibody		2 Antibody	LGS	Tracer Buffer		
	(ul)	(ul)	(ul)	(ul)	4°C., 72Hrs.	(ul)	(ul)	(ul)	4°C., 24Hrs.	- Added 3.0 ml $d.H_2O$ - Centrifuge 4,200 rpm, 45' - Decant the supernatant - Dry the tubes and count
TC	-	-	100	100		-	-	-		
NSB	-	200	100	100*		100	200	-		
Bo	-	200	100	100		100	200	-		
Std	200	-	100	100		100	200	-		
Serum	100	100	100	100		100	100	100		

* = Antibody Buffer is added instead of First Antibody.

Validation and Quality Control

The anti-hCG serum was used at the final dilution of 1 : 16000, which gave 15 - 18 % binding with monkey LH tracer. The sensitivity of the assay when expressed as the lowest dose of the linearized standard curve, was 1.25 ug./ml.. The mean index of precision for 20 multiple-point parallel line assay was 0.06 (Khan et al, 1984).

Culculation

In all assays, NSB counts were interpreted as the percentage of Bo counts and were subtracted from all values counted from the Standards and Samples before a calculation of results takes place.

The percentage of binding of each assay was calculated from

$$\frac{\text{(Mean Bo counts - Mean NSB counts)}}{\text{Mean Tc counts}} \times 100$$

When the value of the quality control pool of that assay deviates greatly from the norm or when the sample determined value falls in the extreme segments of the standard curve where interpolation was deemed unreliable or when values were completely out of the standard curve, the affected samples were re-assayed.

For each assay, a standard curve was drawn on a semi-log graph paper with an ordinate scaled as CPM. or bound form or as percentage of Radioactivity bound (% B/Bo). Bo = B_o counts and B = each sample or standard count - NSB count.

Counter

Progesterone and Estradiol assays were counted by a Packard Tri-carb. Beta counter (Packard Instrument Co., USA).

Prolactin and LH assays were counted by a Gamma counter.