Chapter 2

MATERIALS AND METHODS

1. Chemicals

Sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate and sodium chloride were analytical reagent from Mallinckrodt Chemical Works, St. Louis, U.S.A.

Ether, anhydrous A.R. and benzene, nanograde from Mallinckrodt Chemical Works, St. Louis, U.S.A.

Toluene and dioxane were "Baker Analyzed" from J.T. Baker Chemical Co., Phillipsburg, N.J.

Liquiflour and progesterone-1,2-^{3}H (S.A. = 50 Ci/mM) were purchased from the New England Nuclear Corporation, 575 Albany Street, Boston, Mass. 02118.

Gelatin and ethanol (reagent grade) from BDH Laboratory Chemical Division England.

Sodium azide, laboratory reagent from Hopkins and Williams Ltd., Chadwell Heath Essex England.

Norit A charcoal from Matheson Coleman and Bell, Division of Matheson Company, Inc.; Norwood (Cincinnati), Ohio; East Rutherford, N.J.

Dextran T-70 from Mann Research Labs, Pharmacia, Fine Chemicals AB, Uppsala, Sweden.

Standard progesterone from Sigma Chemical Company, St. Louis, U.S.A.

Progesterone antibody was kindly supplied by Dr George Mikhail, Department of Obstetrics and Gynecology, School of Medicine, University of Pennsylvania, Philadelphia, U.S.A.

2. Instruments and apparatus

1. Packard Tricarb Liquid Scintillation Spectrometer Model - 3390, Packard Instrument Company Inc., Illinois, U.S.A.
2. Refrigerated Centrifuge MSE Model MISTRAL 4L
3. Vortex Mixer
4. Drying Manifold
5. Plastic Racks for tubes insert
6. 10x75 mm test tubes from Kimble Products, Vineland, N.J.
7. 50 ml glass tubes from Arthur H. Thomas Company, Philadelphia, U.S.A.
8. Counting vials which were classified into 3 kinds:

8.1 Low count vials which included ether residue vials, blank recovery vials and sample recovery vials. These vials were reused for ether extracting vials, recovery vials and total recovery vials.

8.2 High count vials which included total cpm recovery vials (1,000 cpm), sample assay vials, standard assay vials and blank assay vials. These vials were reused for assay vials and total recovery vials.

8.3 Very high count vials which included total cpm assay vials (more than 7,000 cpm), vials used in checking 10,000 cpm and those which were used in working dilution of prepared 10,000 cpm/0.1 ml. These very high counting vials were discarded directly into radioactive waste cans after emptying the contents.

3. Subjects

Blood samples from various groups of subjects were kindly supplied by Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn Hospital.

For normal menstrual cycle study, blood samples were obtained from four subjects aging from 18-35 years old and were collected for three consecutive months.

Ten cases of women who used 0.03 mg/day of Norgestrel as contraceptive drugs were studied, four of them supplied their blood samples for 3 cycles - cycles 2, 6 and 12 after Norgestrel treatment; another four supplied their blood samples for 2 cycles - cycle 2 and 6 after treatment and the rest two supplied only blood sample of cycle 2 after treatment.

For pregnancy samples, blood samples were collected from 4-40 weeks of gestation from twenty-nine subjects.

Postpartum blood samples from nine subjects were also collected ranging from 17 to 116 hours after delivery.

4. Reagents

4.1 Assay buffer (0.1 M phosphate buffer, 0.14 M NaCl, 0.015 M sodium azide containing 0.1% gelatin)

The buffer was composed of 32.7 grams of sodium phosphate dibasic heptahydrate, 10.8 grams of sodium phosphate monobasic monohydrate, 2.0 grams of sodium azide, 18.0 grams of sodium chloride and 2.0 grams of gelatin in 2 litres of water. (The conductivity of the water used was less than 1 μ Mho and the pH was 7.0 ± 0.1).

4.2 Charcoal suspension

0.625 grams of Norit A charcoal, 0.0625 grams of dextran T-70 and 100 ml of assay buffer were added into a 100 ml beaker and were mixed vigorously using magnetic stirer.

4.3 Radioactive progesterone

2 µCi (40 µl) of a stock solution of ^{3}H-progesterone (50 µCi/ml absolute ethanol) was pipetted into a clean counting vials and dried down under nitrogen gas. Then 20 ml of assay buffer were added and mixed well. The solution was left to stand at room temperature for at least 1 hour before used. A volume of 0.1 ml which was used in the assay should contain approximately 10,000 cpm.

4.4 Standard progesterone

Stock solution = 1 µg standard progesterone in 1 ml absolute ethanol.

Solution A (1000 pg/0.5 ml) : 20 µl (20 ng) of stock solution + 10 ml of buffer

Solution B (500 pg/0.5 ml) : 5 ml of solution A + 5 ml of buffer

Solution C (250 pg/0.5 ml) : 5 ml of solution B + 5 ml of buffer

Solution D (100 pg/0.5 ml) : 4 ml of solution C + 6 ml of buffer

Solution B (50 pg/0.5 ml) : 5 ml of solution D + 5 ml of buffer

Solution F (25 pg/0.5 ml) : 5 ml of solution E + 5 ml of buffer

Solution G (10 pg/0.5 ml) : 4 ml of solution F + 6 ml of buffer

Solution H (5 pg/0.5 ml) : 5 ml of solution G + 5 ml of buffer

Solution I (2.5 pg/0.5 ml): 5 ml of solution H + 5 ml of buffer

Solution J (0 pg/0.5 ml) : 10 ml of buffer

4.5 Antibody

The antibody used in the study was a gift from Dr G. Mikhail, Department of Obstetrics and Gynecology, University of Pennsylvania and was prepared as followed.

11α - hydroxy hemisuccinate supplied by the Upjohn Company, Kalamazoo, Michigan. This steroid was conjugated to crystalline bovine serum albumin by the mixed anhydried technique (Erlanger, 1957). Three mg of the antigen were dissolved in 1 ml of 0.15 M NaCl. An equal volume of complete Freund's adjuvant was added. The mixture was emulsified and injected to each of two adult ewes via multiple intradermal injections in the axillary and groin regions. This procedure was repeated weekly for six weeks and monthly thereafter. The antibody used in this study was obtained from an ewe 6 months after the initial immunization.

The 1:100 dilution of antibody was prepared by mixing 250 μl of undiluted antiserum with 25 ml assay buffer.

This diluted antiserum can be stored at -20°C for 6 months.

The working diluted solution (concentration 1:4,000) was made by mixing 0.5 ml of 1:100 diluted antiserum with 19.5 ml assay buffer. Under the assay condition, 0.7 ml incubation volume, the final dilution of the antiserum should be 1:28,000.

4.6 Counting solution

640 ml of liquiflour and 3,000 ml of dioxane were added into a drum containing 4 gallons of toluene and mixed well.

4.7 Hormone-free serum

The hormone-free serum was obtained by the following procedure.

Charcoal (100 grams) was added into 500 ml of distilled water, then the mixture was shaken and left for 5 minutes. The fine particles in the supernatant were decanted. This process was repeated for 4 to 5 times. Approximately 60 grams of "washed charcoal" was obtained and spread in an aluminium tray and activated in an oven of not more than 100°C overnight (18 hours at least).

Activated charcoal was mixed with 0.1% sodium azide in serum (50 mg charcoal/1 ml serum) and stirred for 24 hours at

room temperature then centrifuged and filtered through Whatman paper # 42 for several times until a satisfactory clear greyish color of hormone-free serum was obtained.

5. Experimental Procedures

5.1 Serum preparation

Blood samples were left for 2 hours at room temperature and then centrifuged at 3,000 rpm for 10 minutes to remove red blood cell clot from serum.

The following extracting procedure was adopted:

a) A clean 50 ml glass tube was prewashed with ether and 0.1 ml of assay buffer containing 1,000 cpm of radioactive progesterone was added.

b) 1 ml of serum was added to the above.

c) The mixture was mixed and equilibrated at room temperature for 30 minutes.

d) Under a hood with a fan on, 10 volumes of cold ether was added to the serum.

e) The mixture was then mixed in vortex mixer for 30 seconds and left to stand for 5 minutes to allow separation of the two phases.

f) The bottom layer (serum) was frozen by dipping for 1 minute in ethanol containing chips of dry ice.

g) The ether was decanted into "low count" scintillation vials and was dried under filtered air.

h) 1.7 ml of assay buffer was added to the dried residue and mixed well in a vortex mixer.

i) The solution was left to stand at room temperature for 1-2 hours.

j) Two portions (2x0.5 ml) were pipetted into two 10x75 mm test tubes and assayed as described under "assay procedure" page 27 .

k) Another 0.5 ml portion was pipetted into "low count" counting vials and 10 ml of counting solution was added, mixed well and counted for 10 minutes. This was to determine the recovery of the extracted serum.

5.2 Assay procedure

The incubation was performed in a total volume of 0.7 ml and three sets of assay tubes were simultaneously prepared. The constituents in each set were shown below.

		Blank	Standard	Samples
assay buffer	(ml)	0.6	-	-
serum extract in assay buffer	(ml)	-	-	0.5
standard progesterone (A-J, page 23)	(ml)	-	0.5	-
^{3}H-progesterone (= 10,000 cpm)	(ml)	0.1	0.1	0.1
antibody	(ml)	-	0.1	0.1

The incubation mixtures were incubated at appropriate temperature for a required length of time and then the free and bound hormones were separated as described under 5.3.

5.3 Separation of free from bound progesterone

The following procedure was adopted for separating the free from bound progesterone:

a) The charcoal suspension and assay racks were kept at 0-4°C in an ice bath.

b) The charcoal were stirred for 15 minutes.

c) The charcoal suspension (0.2 ml, page 23) was pipetted into every tube in the assay rack, and shook for 3 times.

d) The mixture was left to stand at 0-4°C for 20 minutes and centrifuged at 2,500 rpm for 15 minutes.

e) The supernatant from each tube were decanted into "high count" vials.

g) Ten ml of counting solution was added into each vial and mixed well. The mixture was equilibrated for 30 minutes and counted for 2 minutes.

5.4 Measurement of estradiol

Estradiol level in women during contraceptive treatment were determined by the method of Abraham (1971). This part of the study was carried out by Mr Smai Leepipatpaiboon, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn Hospital.

5.5 Calculations

5.5.1 Standard curve

a) B/B_o of each concentration of the standard progesterone (A-J) was calculated. (B = labelled progesterone bound to antibody expressed as cpm/tube; B_o = amount of labelled progesterone bound to antibody in absence of unlabelled progesterone)

b) The mean $\pm$ S.D. of each B/B_o was then calculated and plotted versus the log amount of standard progesterone added.

c) The sensitivity of the standard curve was calculated by substracting 2xS.D. from the mean cpm bound and divided the result by the same mean cpm bound value at the zero point. The corresponding dose of progesterone was the sensitivity.

d) The precision of the standard curve was estimated in two ways - within-assay and between-assay precision. The within and between-assay variance were determined by duplicate measurements of the same samples for three consecutive days. The coefficient of variation (cv) was calculated by the following formula (Abraham et al, 1971):

$$cv = \sqrt{\frac{\Sigma d^2}{2n}}$$

where $d = \frac{\text{highest value of each duplicate}}{\text{lowest value of same duplicate}} - 1 \times 100$

n = number of duplicate determinations.

5.5.2 Blanks

a) The values of B/B_o of the four blanks which run in triplicate were calculated and taken to interpolate on the standard curve. The corresponding doses of progesterone were the blank values.

b) If the blank values were above the sensitivity of the standard curve. The mean of the blank values had to be substracted from the values of the unknown samples.

5.5.3 Unknown samples

a) The values of B/B_o of the unknown samples were calculated and taken to interpolate on the standard curve.

b) The mean blank was substracted from the unknown samples as mentioned above.

c) The recovery was corrected by dividing (b) by cpm recovered and multiplied by the total cpm added for recovery estimation.

d) The results obtained was considered as the progesterone values present in the unknown samples.

Experimental Protocol

The investigation can be divided into 2 parts:

1. Standardization of a radioimmunoassay of serum progesterone using antiserum obtained by the immunization of an ewe with 11α - hydroxy progesterone hemisuccinate conjugated to bovine serum albumin. A charcoal adsorption technique was applied for the separation of free and bound hormone.

2. The application of the standardized method for the studies of serum progesterone levels in various physiological status - normal and contraceptive menstrual cycle, normal pregnancy and postpartum period.