CHAPTER III

MATERIALS AND METHODS

Materials

1. Animal

Fifteen male cynomolgus monkeys (*Macaca fascicularis*) were randomly selected from 40 male monkeys in the Primate Breeding Colony of Biology Department, Chulalongkorn University. These monkeys were originated from Don Hoi Lord, Samut Songkram Province and borned in the Breeding Colony except no.704, 93 and 56. (Table 1).

They were housed individually in each galvanized iron cage. The photoperiod was 0600-1800 h light. Temperature and humidity were slightly fluctuated according to the season. The animals were fed daily in the morning with monkey chow (purchased from Pokphan Animal Feed Co., Ltd., Thailand) and supplemented in the afternoon with fresh fruits, vegetables and occasionally chicken boiled eggs.

2. Chemical Reagents and Instruments

See appendix I, II and III

Table 1 History and administration doses of morphine hydrochloride in studied male cynomolgus monkeys.

Group of animal	Monkey no.	Age at study (yr/mth)	B.W. (kg)	Dose of morphine (mg/kg/day)
1. Pubertal Monkeys				
	522	5/4	6.00	3.0
	523	4/0	5.75	3.0
	524	4/0	5.20	3.0
2. Adult Monkeys				
2.1)	93	13/5	8.90	1.5
	504	13/0	5.80	1.5
	507	12/0	6.05	1.5
	512	11/1	6.80	1.5
2.2)	505	11/8	7.20	3.0
	509	10/11	8.70	3.0
	511	10/3	6.75	3.0
2.3)	506	12/0	6.50	6.0
	508	11/11	6.50	6.0
	704	12/6	5.60	6.0
3. Untreated Control Monkeys				
3.1) pubertal control				
	525	4/1	5.45	-
3.2) adult control				
	56	13/8	8.1	-

Methods

1. Dosages and treatment schedules

1.1 The study of chronic effect of morphine

From the female data (Settheetham, 1992) the lowest effective dose of morphine in long-term treatment on the turnover rate was 1.6 mg/kg/day. However it has been noticed that the males has a higher threshold and lower sensitivity to the drug treatment (Goldstein et al, 1974). Therefore, in this study, the lowest dose of morphine administration was 1.5 mg/kg/day and duplicated to 3.0 and 6.0 mg/kg/day respectively.

Animals were divided into 3 experimental groups :

1) Pubertal monkey group

The pubertal stage in male cynomolgus monkey indicated by descent of the testes into the scrotal sac (Steiner and Bremner, 1981), and in some monkeys the sexual skin became a reddish colour (Sade, 1964)(Figure 6). Usually in male cynomolgus monkeys, the begining age of pubertal males was about 4.0 years (Varavudhi et al., 1982). Treatment schedule was separated into 3 periods ; pretreatment, treatment and posttreatment periods.

-Pretreatment period

Animals were administered 0.5 ml physiological saline at 1200-1300 h by subcutaneous route for 24 days as a placebo for regard the effect of injection on hormonal changes in treatment period. Four milliliters of blood sample was collected on the second day of saline injection and following with once a week collection.

The turnover rate study was started on the third day of saline injection and measured ten days interval thereafter.

-Treatment period

Morphine hydrochloride solution in dose 3.0 mg/kg/day was subcutaneously injected to monkeys at 1200-1300 h for 74 days. The first blood collection of treatment period was taken during 20 hours after morphine injection (at 0800-0900 h of the second day of treatment period). After that, blood collection and the turnover rate determination were intermitted as pretreatment period.

-Posttreatment period

Animals were treated with 0.5 ml physiological saline as pretreatment period. Since the morphine turnover in female cynomolgus monkey treated with 1.6 and 3.2 mg/kg/day morphine showed a sudden recovery after the drug withdrawal (Setheetham, 1992). In order to follow this phenomena in male monkey, the turnover rate was arranged to measure on the second day and the blood collection was moved to the third day of posttreatment period.

2) Adult monkey group

In the case of male cynomolgus monkey, testosterone level reached a consistent plateau at about 5.0 years old which was a sign of adult monkey. At the onset of the study, the age of adult males averaged 11.75 $\pm$ 0.33 years. Monkeys were divided into 3 sub-group, each group included 4, 3 and 3 monkeys and treated concomitantly with 1.5, 3.0 and 6.0 mg/kg/day morphine hydrochloride, respectively. The schedule of treatment was quite different in each

sub-group. In adult males treated with 3.0 mg/kg/day morphine it was similar with in pubertal group so that the data between these groups could be compared in response for the same dose of morphine. Therefore, the remaining explanation schedule was used for the sub-group of animals treated with 1.5 and 6.0 mg/kg/day morphine hydrochloride only.

-Pretreatment period

Monkeys were injected 0.5 ml saline at 1200-1300 h for 30 days. Blood samples were collected periodically in every week and the turnover rate was determined in every ten days, respectively.

-Treatment period

Monkeys were administered morphine hydrochloride solution by subcutaneous injection in doses 1.5 and 6.0 mg/kg/day by the duration of 130 days and 110 days in each group, respectively. The time intervals of blood collection and turnover rate determination were the same as pretreatment period.

-Posttreatment period

Saline was injected for 63 and 58 days after drug withdrawal in each sub-group that was treated with 1.5 and 6.0 mg/kg/day morphine, respectively. Blood collection and turnover rate study were intermitted as pretreatment period.

3) Control group

Normal puberty and adult male monkeys were selected to collect the blood sample following treatment groups

schedules. They were daily injected with 0.5 ml physiological saline throughout the onset of study. The turnover rate determination was omitted.

All blood samples were analyzed for PRL, TSH, T_4, E_2, T and cortisol concentrations by radioimmunoassay techniques.

The studied schedules were shown in figure 7.

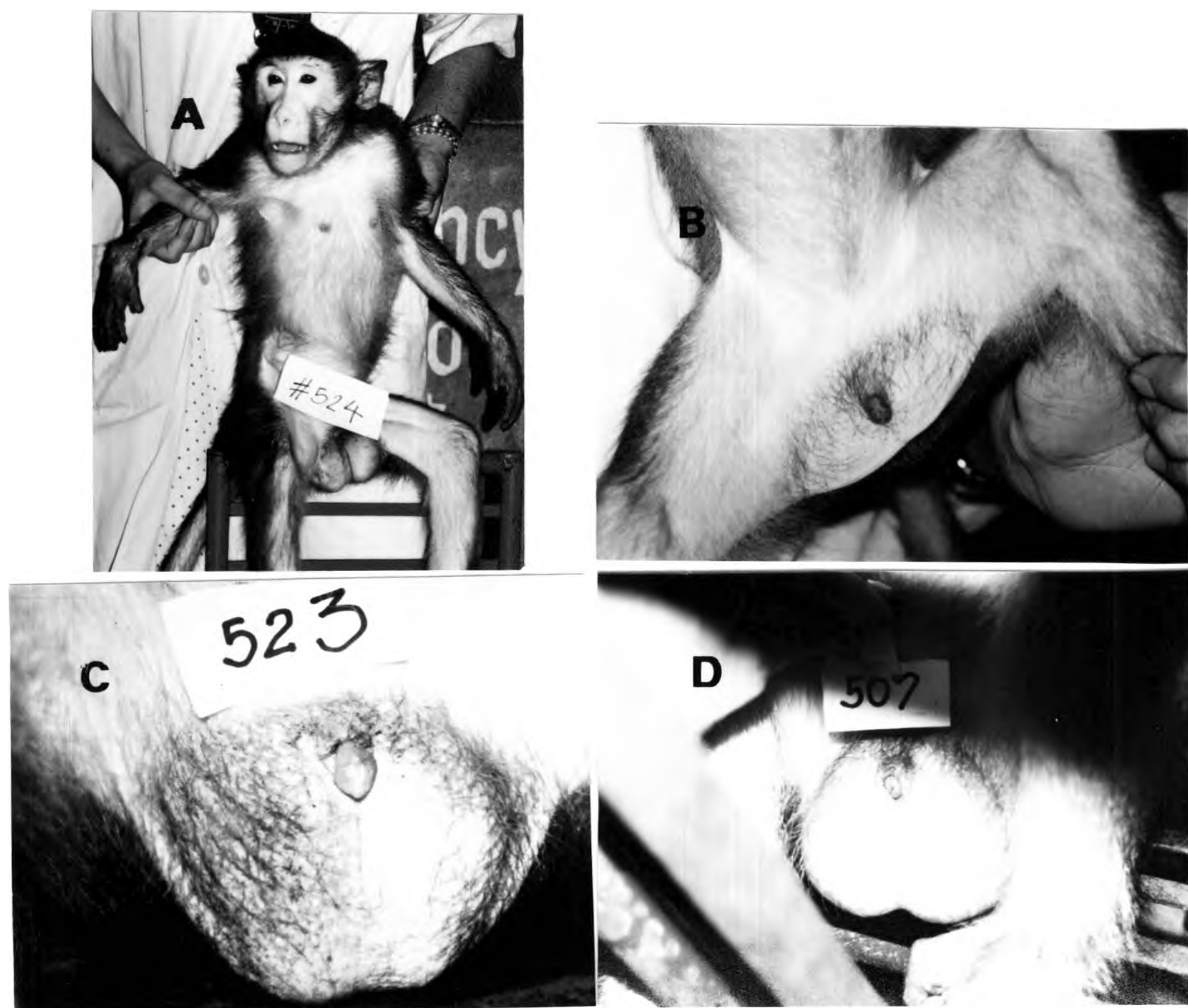

Figure 6 A)The pubertal male cynomolgus monkey at 4.0 years old. B)The appearance of reproductive organs during infancy stage (19 months). C)Testicular descent during pubertal stage (at 4.0 years old) D)The increase in testicular size with a reddish scrotal skin in adult stage.

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
MAY																															*
JUN	B *	T *	*	*	*	*	*	B *	*	*	*	T *	*	*	B *	*	*	*	*	*	*	T *	B *	∴	B ∴	T ∴	∴	∴	∴	∴	X
JUL	∴	B ∴	∴	∴	∴	T ∴	∴	∴	B ∴	∴	∴	∴	∴	∴	∴	B ∴	T ∴	∴	∴	∴	∴	∴	B ∴	∴	∴	T ∴	∴	∴	∴	B ∴	∴
AUG	∴	∴	∴	∴	T ∴	B ∴	∴	∴	∴	∴	∴	∴	B ∴	∴	T ∴	∴	∴	∴	∴	B ∴	∴	∴	∴	∴	T ∴	∴	∴	∴	∴	B ∴	∴
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OCT	*	*	*	*	*	B *	T *	*	*	*	*	*	B *	*	*	*	T *	*	*	B *	*	*	*	*	*	*	T *	B *			

1992

B

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APR																											*	B *	T *	*	X
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JUL	∴	B ∴	∴	∴	∴	∴	∴	T ∴	B ∴	∴	∴	∴	∴	∴	∴	B ∴	∴	T ∴	∴	∴	∴	∴	B ∴	∴	∴	∴	∴	T ∴	∴	B ∴	∴
AUG	∴	∴	∴	∴	∴	B ∴	T ∴	∴	∴	∴	∴	∴	B ∴	∴	∴	∴	T ∴	∴	∴	B ∴	∴	∴	∴	∴	∴	∴	B ∴	T ∴	∴	∴	∴
SEP	∴	∴	B ∴	∴	∴	T ∴	∴	∴	∴	B ∴	∴	∴	∴	∴	∴	T ∴	B ∴	∴	∴	∴	∴	∴	∴	B ∴	∴	T ∴	∴	∴	∴	∴	X
OCT	B ∴	∴	∴	*	B *	T *	*	*	*	*	*	B *	*	*	*	T *	*	*	B *	*	*	*	*	*	*	B *	T *	*	*	*	*
NOV	*	B *	*	*	T *	*	*	*	B *	*	*	*	*	*	T *	B *	*	*	*	*	*	*	B *	*	T *	*	*	*	*	B *	X
DEC	*	*	*	*	T *																										

1993

C

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
APR																													*	B *	X
MAY	T *	*	*	*	*	*	B *	*	*	*	T *	*	*	B *	*	*	*	*	*	*	B *	T *	*	*	*	*	*	B *	∴	B ∴	T ∴
JUN	∴	∴	∴	∴	∴	B ∴	∴	∴	∴	T ∴	∴	∴	B ∴	∴	∴	∴	∴	∴	∴	B ∴	T ∴	∴	∴	∴	∴	∴	B ∴	∴	∴	T ∴	X
JUL	∴	∴	∴	B ∴	∴	∴	∴	∴	∴	T ∴	B ∴	∴	∴	∴	∴	∴	∴	B ∴	∴	T ∴	∴	∴	∴	∴	B ∴	∴	∴	∴	∴	T ∴	∴
AUG	B ∴	∴	∴	∴	∴	∴	∴	B ∴	T ∴	∴	∴	∴	∴	∴	B ∴	∴	∴	∴	T ∴	∴	∴	B ∴	∴	∴	∴	∴	∴	∴	B ∴	T ∴	∴
SEP	∴	∴	∴	∴	B ∴	∴	∴	T ∴	∴	∴	∴	B ∴	∴	∴	∴	*	B *	T *	*	*	*	*	*	B *	*	*	*	T *	*	*	X
OCT	B *	*	*	*	*	*	*	B *	T *	*	*	*	*	*	B *	*	*	T *	*	*	*	B *	*	*	*	*	*	T *	B *	*	*
NOV	*	*	*	*	B *	*	T *	*	*	*	*	B *																			

1993

Figure 7 The study protocol in A) adult and pubertal monkeys treated with 3.0 mg/kg/day morphine hydrochloride, B) adult monkeys treated with 1.5 mg/kg/day morphine hydrochloride and C) adult monkeys treated with 6.0 mg/kg/day morphine hydrochloride, respectively.

Abbreviation : * : 0.5 ml saline injection
∴ : morphine hydrochloride administration
B : blood sample collection
T : turnover rate determination

1.1.1 Drug administration

All monkeys were injected subcutaneously with saline or morphine hydrochloride solution at 1200-1300 h around the ischial callosities areas of the buttock which has a thick adipose tissue layer (Hartman and Straus, 1971). For avoiding the risk of deeper injection that means muscular route, the needle no.26G x 1/2" was used. However, the pharmacokinetics effect of morphine by subcutaneous and muscular route were not so particularly different (Brunk and Delle,1974; Iwamoto and Klaassen, 1977).

It is keeping in mind that morphine depresses respiration, principally by reducing the sensitivity of the brain stem respiratory centers to arterial carbon dioxide tension ($PaCO_2$) (Adrini, 1970). Initially, respiratory rate is affected more than tidal volume, but as the dose of morphine is increased, periodic breathing and apnea occur. It is important not to use other respiratory depressants, e.g., anaesthetic drugs, with morphine until the full extent of the morphine respiratory depression is manifest, more than 0.5-1 hour when it has been given by intramuscularly or subcutaneously (Wood-Smith and Steward, 1964; Wood and Wood, 1982). Therefore, anaesthetization with ketamine hydrochloride which its effect of hypoxic or hypercarbic stimulation of respiration is not seriously affected following usual doses, should be used with great caution in any situation of morphine treatment (Gilman et al., 1985). Additionally, to observe the responsiveness to any drugs the discriminated times of injection were used. At 0800-0900 h, all monkeys were anaesthetized with ketamine hydrochloride before each blood collection and at 1200-1300 h they were administered either

saline or morphine solution.

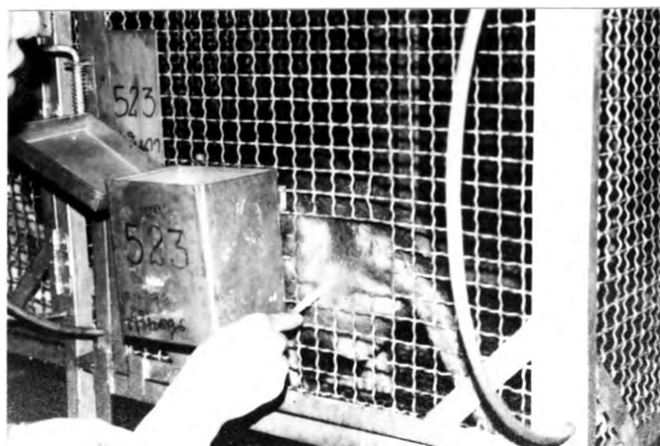

Figure 8 Subcutaneous injection of morphine hydrochloride around the ischial callosities areas.

1.1.2 Blood collection

Fasting blood sample were drawn from the anaesthetized male monkeys which were kept in restraining chairs (Figure 9). The anaesthetic drug was ketamine hydrochloride using in the dose 5.0 mg/kg i.m. or lower depending on the experience in each monkey. The restraining chair and monkey cages were placed in a same room for minimizing environmental changes and stressful effects. To minimize the effect of possible diurnal fluctuations in endogenous hormone release, all blood samples were collected between 0800-0900 h by femoral venipuncture. Blood serum was separated immediately after the blood clotting at the room temperature by centrifugation at 1000 xg 20 minutes. Serum was then aliquoated and stored at -20 ^{o}C until hormonal assays by RIA techniques. All samples from an individual subject for any given hormone were run in the same assay.

Figure 9 Adult male monkey keeping in the restraining chair.

1.1.3 Metabolic turnover rate study

The metabolic turnover rate is the rate of drug degradation and excretion in the whole body (Goodman and Noble, 1968). The concentration of drug in the systemic circulation will be related to the concentration of drug at its site of action. The clinician usually wants to maintain steady-state concentrations of a drug within a known therapeutic range. Therefore, the elimination rate (rate for metabolism and excretion of the drug) is the most important concept to be considered when a rational regimen for drug administration is to be designed (Gilman et al., 1985).

For turnover rate study, 0.5 ml radioactive morphine solution was administered by a bolus intravenous injection into saphenous vein of male monkeys. Then, 1 ml venous blood samples

were taken frequently at 5, 15, 30, 60, 120, 240, and 360 minutes from the radial vein into the heparinized syringes. Blood plasma was separated by centrifugation at 1000 xg 20 minutes. Four hundreds microliters blood plasma was pipetted into a counting vial and reconstituted with scintillation fluid. The mixer was then counted in the beta counter at least 1 hour to obtain 10,000 cpm count of the blood plasma collected at 5 minutes.

The semilogarithmic plot of cpm count versus time appeared to indicate that morphine decreased in a manner of multiexponential kinetics in male cynomolgus monkeys. The point concentrations in plasma were fitted to a biexponential equation after intravenous doses.

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

This function is consistent with standard two-compartment open pharmacokinetic model. The quantity C represents plasma morphine concentration at time t after the end of the injection. The coefficients A, B and the exponents α , β are hybrid quantities. The y-intercept of the two straight lines directly provide the value of the constants A and B. The constants α and β which are the rate constants for distribution and elimination, respectively can be directly calculated from slopes of the two straight lines (Figure 10). Therefore, the values of these constants can be determined from the half-lives of the two equations (Stanski, Greenblatt and Lowenstein, 1978; Gilman et al., 1985)

$$\alpha = 0.693 / t_{1/2\alpha}$$

$$\beta = 0.693 / t_{1/2\beta}$$

While, $t_{1/2\alpha}$ is an apparent distribution half-life

$t_{1/2\beta}$ is an apparent elimination half-life

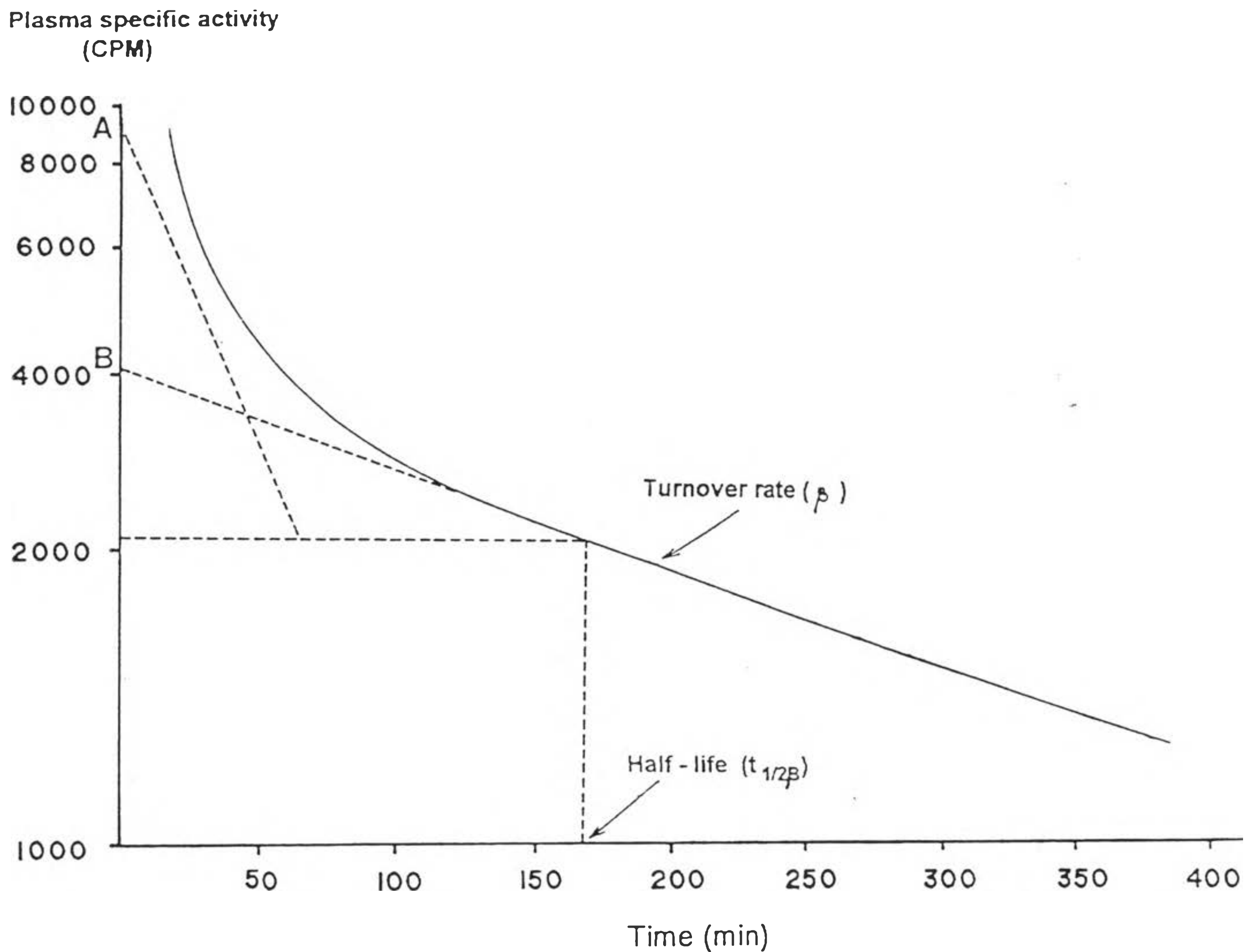

Figure 10 Plasma cpm count-time curve following intravenous administration of radioactive morphine solution to male cynomolgus monkeys.

1.1.4 Testicular measurements

Testicular measurements were obtained with a sliding caliper, in a manner similar to that used by Sade(1964). The time of measurements carried out during the blood sample collections. To eliminate inclusion of the epididymis and surrounding tissue, the scrotal sac was stretched during measurement. Testis size was taken to be length plus breadth on the left testicle only to account for possible influence of bilateral asymmetry. Length is the longest diameter of the testis parallel to, but excluding, the epididymis. Breadth is the greatest lateral-medial diameter and also excluded the epididymis (Figure 11).

The average of measurements in every months were also calculated in correlation with age and body weight.

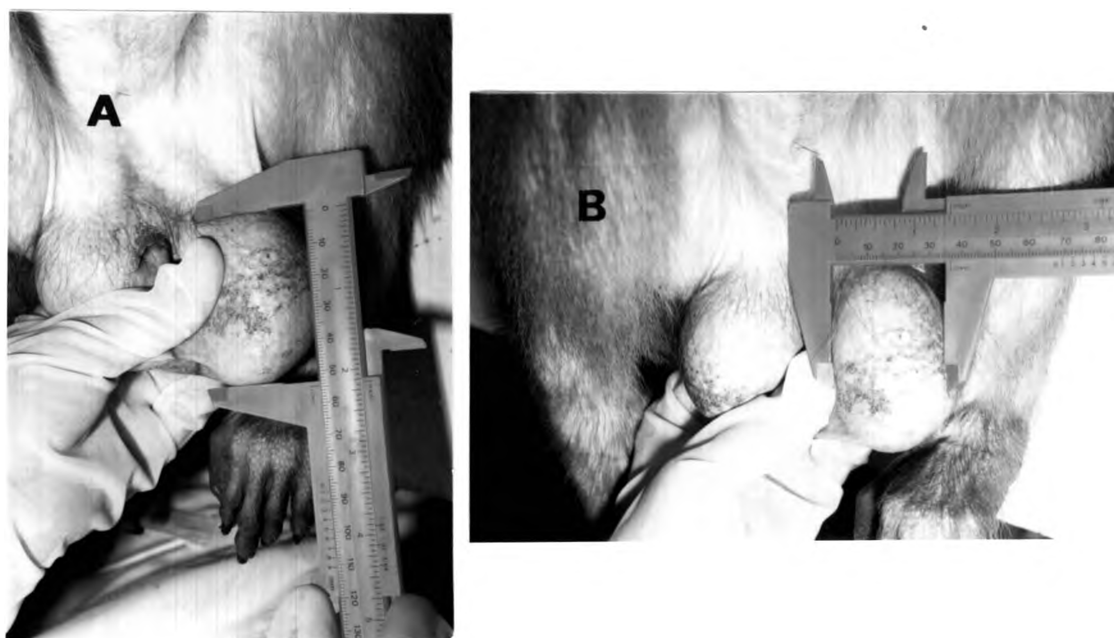

Figure 11 Testicular size was taken to be length (A) plus breadth (B) on the left testis.

1.2 The study of acute effect of morphine

From the long-term treatment of morphine, hormonal analyses of blood samples collected within 20 hours after the drug injection was unable to compare with results of long-term morphine treatments in female cynomolgus monkeys from which blood samples were collected approximately 1 hour after daily morphine administration (Settheetham, 1992). In order to assess if morphine exert an adverse effect to any hormonal levels earlier than 20 hours, thirteen male monkeys which were studied in the previously described experiment were divided into 4 groups and treated the same doses of morphine as the study of chronic effect. The study has been done after several months of morphine withdrawal. This acute study also compared with normal adult monkeys (no.519 and 520) whom never have experiences of morphine treatment before. They were treated with morphine hydrochloride in dose 3.0 or 6.0 mg/kg.

All monkeys were anaesthetized with ketamine hydrochloride before the first blood withdrawal for keeping the monkey condition as in the chronic effect study. One-milliliter blood samples were taken frequently at -1, -0.5, 0, 0.25, 0.5, 1, 1.5, 2.5, 4.5, 6.5 and 10 hours of subcutaneous morphine injection from the radial vein of the monkeys restrained in a squeeze cage. Blood serum was separated immediately after the blood clotting at the room temperature by centrifugation at 1000 xg 20 minutes. Samples from each subject were analyzed for PRL, TSH, T_4, testosterone and cortisol levels in the same assay to minimize interassay variation.

Table 2 The administration doses of morphine hydrochloride for acute response in monkeys previously studied in the chronic effect.

Monkey no.	Morphine dose (mg/kg)	B.W. (kg)	Total dose (mg)	Day after morphine withdrawal
1. Pubertal Monkeys				
522	3.0	7.2	21.6	457
523	3.0	6.5	19.5	459
524	3.0	5.6	16.8	459
2. Adult Monkeys				
93	1.5	9.2	13.8	68
504	1.5	5.8	8.7	68
507	1.5	7.1	10.6	68
512	1.5	7.2	10.8	67
505	3.0	6.8	20.4	456
509	3.0	7.6	22.8	454
511	3.0	6.7	20.1	457
506	6.0	6.9	40.4	99
508	6.0	6.6	39.6	85
704	6.0	4.9	29.4	69

2. Hormonal determinations

Serum samples were analyzed for hormonal levels of TSH, PRL, T_4, E_2, testosterone and cortisol by using radioimmunoassay techniques (RIA). The reliability of RIA is assessed by four main criteria : specificity, sensitivity, precision and accuracy (Chard, 1978; Thorell and Larson, 1978).

Specificity :

Specificity is the ability of the assay to measure one specific compound and no other. The identifiable material with physicochemically similar to the ligand that can interfere the assay by reacting directly with the binder is called a cross-reacting material. The most common way of presenting the specificity is to compare the amount of the cross-reacting material under study which yields 50 percent inhibition of binding (x) with the amount of standard giving the same inhibition (y), and then to express the potency of the cross-reacting material as a percentage of the standard.

$$\text{Percent cross-reactivity} = y/x \times 100$$

Sensitivity :

Sensitivity is defined as the minimal detection limit of an assay. It may refer to the least concentration of unlabelled ligand which can be distinguished from a sample containing no unlabelled ligand. It can be determined base on the confidence limits to the zero standard estimate, on the other hand, the confidence limits to standard estimate. It is that the sensitivity

of an assay is critically dependent on the precision of the assay.

Precision :

Precision which often refers to as reproducibility is a measure of the variation observed between repeated determination of the same sample. It is usually expressed as the coefficient of variation. Intraassay, within-assay, variation refers to precision within an individual assay run and interassay, between-assay, variation refers to precision from different sets of assays of the same sample. For monitoring the precision, the pool sample should be set up and their concentration should represent high, medium and low values in the assay. This provides an on-going check of precision at different parts of the standard curve.

Accuracy :

Accuracy is the degree to which the estimate approximates the true value. It is expressed as the correlation coefficient between the determined and added values.

Prolactin(PRL) assay

In 1971, Hwang, Guyda and Friesen successed in extraction of human prolactin (hPRL) from fractions of pituitary extracts obtained from the purification of growth hormone. Therefore, the development of RIA for hPRL has been demonstrated (Guigley and Haney, 1980) and is now readily available as many kits. Azukizawa, et al.(1976) and Melmed, et al.(1979) evaluated monkey PRL level by the double antibody method using the NIAMDD kit for human PRL or using human PRL as standard (Wardlaw et al., 1980). At here, PRL

levels of male cynomolgus monkey (mPRL) were determined by using the homogenous radioimmunoassay. These homogenous PRL assay (L' Hermite, 1973) using monkey sample competed with hPRL to antiserum of hPRL. Quadri and Spies (1976) presented the parallelism in the immunoreactivities of human PRL standard with PRL of rhesus monkey from pituitary extract, postpartum plasma and purified monkey PRL. PRL assay in this procedure followed the Diagnostic Products Corporation (DPC), USA and latter modified by using the half reduction of sample and reagents volume.

Reagents preparation :

1. Prolactin antiserum

The antiserum to hPRL has been produced in the rabbit and provided in a lyophilized from. It has been reconstituted by adding 10 ml distrilled water and mixed by gentle version. After reconstitution the solution could be stable at 2-8 ^{o}C for at least 30 days.

2. ^{125}I-prolactin

The iodinated hPRL has been purified by affinity chromatography using monoclonal antibodies. It was also supplied in lyophilized from and dissolved by adding 10 ml distilled water. The solution could be stable at 2-8 ^{o}C and to be used until the expiration date marked on the vial.

3. hPRL standard

The standards have been prepared in human serum stripped of PRL by affinity chromatography. They represent 0, 5, 10,

20, 50, 100 and 200 ng/ml in terms of WHO First International Reference Preparation of hPRL for RIA, no.75/504 (1st IRP 75/504). The term the more recent of Second International Standard for PRL, no.83/562, they have values of 0, 115, 230, 460, 1150, 2300 and 4600 mIU/L (2^{nd}IS 83/562), were used. Intermediate standard points could be obtained by mixing calibrators in suitable proportion. At least 30 minutes prior to use, reconstituted the zero standard with 6.0 ml distilled water and each of the remaining standard with 3.0 ml distilled water. The solutions have been stable at -20 °C for at least 30 days after reconstitution.

4. Precipitating solution

The precipitating solution consisted of goat anti-rabbit gamma globulin (second antibody) and polyethylene glycol (PEG) in saline. The precipitating solution was supplied in liquid form, ready to be used and PEG was helpful for separation of bound form from free form. The reagent stored at 2-8 °C and mixed well before use.

Assay procedure :

One hundreds microliters of zero standard was pipetted into the NSB and B_o tubes and 100 ul of each of the remaining standard, quality control and unknown samples into the corresponding labeled tubes. ^{125}I-PRL 50 ul was added to each tube and gently mixed. Fifty microliters of PRL antiserum was added to all tubes except the NSB and T_c tubes. Incubation was done at room temperature for 3 hours and followed by adding 500 ul of cold and well mixed precipitating solution to all tubes and votexed. The

precipitated bound form was separated from free form by centrifugation at 3000 xg for 15 minutes. The supernatant was decanted and the retaining precipitate of each tube was counted for 1 minute by gamma-counter.

Thyrotropin(TSH) assay

The particular problem of TSH assay was a sufficient sensitivity to detect euthyroid levels of TSH in unextracted serum with optimal precision (Pekary, Hershman and Parlow, 1975). This is due to , in part, TSH exhibited the low concentration (0.5-5.0 uIU/ml in man : Pekary , Hershman and Parlow 1975; 0.2-6.8 uIU/ml in rhesus monkey : Wherry et al., 1970 ; Azukizawa et al., 1976; Melmed et al., 1979 and 6.3 $\pm$ 0.3 uIU/ml in cynomolgus monkey : Ren et al., 1988) of normal thyroid function. Therefore, most TSH kits appeared to have difficulty measuring correctly in the low end of the normal range (Musto, Pizzolante and Chesarrone, 1984). Pekary, Hershman and Parlow (1975) developed a hTSH RIA for improvement in sensitivity and reliability of assay by using of a new high-specificity, high-affinity antibody to hTSH but it still could be cross-reacted with mTSH of rhesus monkey pituitary homogenate. Also, another experiment (Wherry et al., 1970) successed in determination mTSH by means of RIA using the double antibody technique and yet demonstrated a parallel cross-reactivity with hTSH to antibody to human TSH. These leaded many investigators (Azukizawa et al., 1976; Melmed et al., 1979; Ren et al., 1988) measured mTSH by RIA employing the human TSH kit. In this assay, monkey TSH was measured by RIA using the human TSH kit from Instar Corporation, USA and latter minor modification by reduction the sample and reagents volume (Figure 12). The

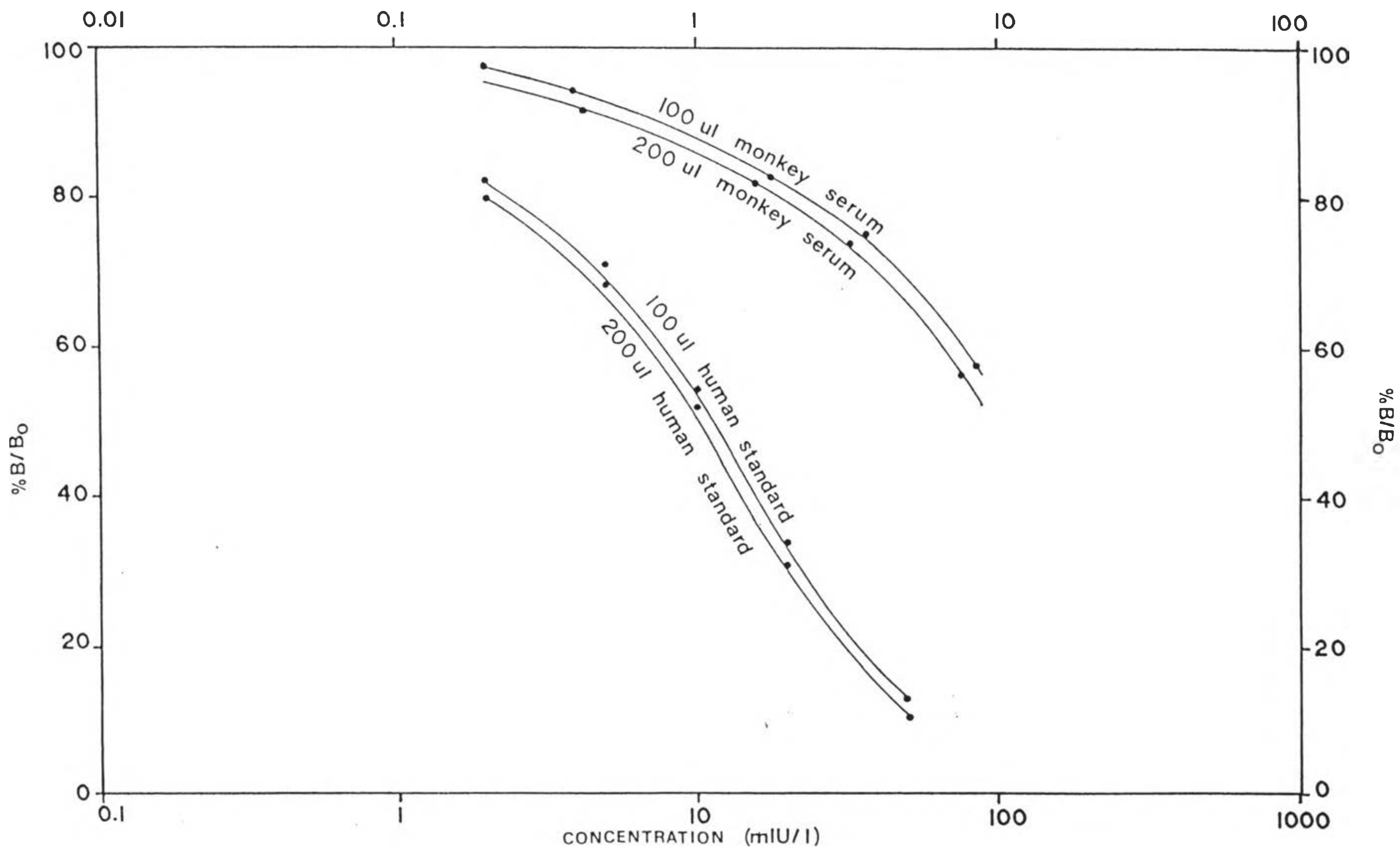

Figure 12 Parallelism check between original protocol and a minor modification protocol by a half reduction volume in hTSH radioimmunoassay.

dilution curve of serum from hypothyroidism monkey containing a high level of TSH was parallel with the standard curve of human TSH (Suwanprasert, 1991).

Reagents preparation :

1. TSH antiserum

TSH antiserum was available for using in liquid form. One vial contained 14 ml of rabbit anti-hTSH serum, normal rabbit serum, gelatin in barbital buffered saline and inert red coloring with 0.02 M sodium azide as a preservative.

2. ^{125}I-TSH

The solution was ready to use consisting of approximately 3 uCi tracer in 5 ml of gelatin in barbital buffered saline and inert blue coloring with 0.002 M sodium azide.

3. hTSH standard

Each vial contained hTSH in 2 ml (except the zero standard containing 4 ml) of barbital saline, bovine serum albumin with 0.02 M sodium azide as a preservative. The standards was calibrated with Medical Research Council (MRC) WHO human pituitary TSH 68/38 at 0, 2, 5, 10, 20 and 50 uIU/ml, respectively.

4. Precipitating solution

Precipitating solution contained 130 ml of goat anti-rabbit serum and a water soluble polymer in phosphate buffer with 0.02 M sodium azide as a preservative. To ensure a homogeneous mixture, it should be mixed throughly before use.

5. hTSH non-specific binding reagent

The term non-specific binding usually denoted the occurrence of adsorption of free radioligand to any kinds of solid material. In some assay, this was easy to perform by not adding any antiserum to the tube and replace by adding non-specific binding reagent (Thorell and Larson, 1978). The solution contained normal rabbit serum, gelatin in barbital buffered saline and inert red coloring with 0.02 M sodium azide as a preservative.

6. hTSH quality control

hTSH controls were calibrated at 3 and 17 uIU/ml, respectively. They contained human TSH in barbital buffered saline, bovine serum albumin with 0.02 M sodium azide as a preservative.

All solutions were kept at 2-8 ^{o}C.

Assay procedure :

One hundreds microliters of hTSH zero standard was aliquoted to the NSB and B_o tubes and 100 ul of each of the remaining standard, quality control and unknown samples into the corresponding labelled tubes in duplicated sets. Fifty microliters of rabbit anti-human TSH serum was added to all tubes, except the T_c and NSB tubes, and the NSB tubes were contained 50 ul of non-specific binding reagent instead, gently mixing each tube. After incubation for 1 hour in a 37 ^{o}C water bath, 50ul of ^{125}I-hTSH was added to all tubes and followed gently mixing each tube. Incubation in a 37 ^{o}C water bath continued for 2 hours, then 500 ul of well-mixed precipitating antiserum reagent was added to all tubes except T_c tubes and vortex mixed. All tubes allowed to incubate at

37 ^{o}C for at least 5 minutes and then centrifuged (except T_C tubes) at 1000 xg for 15 minutes. The supernatants were decanted and trapped the rim of each tube into absorbent paper to remove any adhering supernatant. The precipitated was counted in a gamma counter for 1 minute.

Thyroxin(T_4) assay

Thyroxin concentraton in monkey serum was determined by using human T_4 kit, at first in rhesus monkey (Azukizawa et al., 1976) and then in cynomolgus monkey (Ren et al., 1988). To accurately measure T_4 in unextracted serum it was necessary in the presence of blocking agents to prevent the binding to thyroid hormone-binding protein. This was accomplished by using barbital buffer and 8-anilino-1-nathalene sulfonic acid (ANS) to inhibit binding to thyroxin binding prealbumin (TBPA) and thyroxin binding globulin (TBG), respectively. The polyethylene glycol was preferentially used as an aid for second antibody to separate bound from free form (Mitsuma et al., 1972). This assay procedure followed the modified protocol from Diagnostic Products Corporation (USA) by the PEG-accelerated double-antibody method.

Reagents preparation :

1. T_4 antiserum

The antiserum was highly specific for T_4, with very low crossreactivity to other compounds. Neither protein, lipemia, bilirubin nor hemolysis had any effect on the assay. T_4 antiserum was provided in lyophilized form, reconstitution by adding 10 ml distilled water and mixing by gentle inversion before use. the

solution could not be freezed.

2. $^{125}I-T_4$

$^{125}I-T_4$ was supplied in lyophilized form with the blocking agents to prevent binding of radiolabeled T_4 to thyroid hormone-binding proteins. It was dissolved with 11 ml distilled water before use and stable until the expiration date marked on the vial.

3. hT_4 standard

hT_4 standards contained 0, 1, 4, 10, 16 and 24 ug/dl of T_4 or equivalently to 0, 12.9, 51.5, 129, 206 and 309 nmol/l (multiply by 12.87 for converting from ug/dl to nmol/l). The standards were supplied in liquid form, ready to use.

4. Precipitating solution

One vial of precipitating solution consisting of goat anti-rabbit gamma globulin (second antibody) and diluted PEG in saline, was supplied in liquid form ready to use. Since a fine precipitate could form after refigeration, the precipitating solution was throughly mixed before use, without foaming.

All of reagents could be stable at 2-8 ^{o}C at least 30 days.

Assay procedure :

The assay procedure was performed by the sequential addition of 10 ul of the zero standard into the NSB and B_o tubes

and 10 ul of remaining standard, quality control and unknown monkey serum into the tubes prepared in duplicate, 50 ul of ^{125}I-T_4 and T_4 antiserum to all tubes, except the NSB and T_c tubes for T_4 antiserum. The assay mixture was agitated in a vortex mixer and incubated for 20 minutes at room temperature. After incubation the antibody-bound hormone was separated by the addition of 500 ul of cold precipitating solution. The reaction mixture was agitated in vortex mixer, incubated at room temperature for 5 minutes, and centrifuged at 3000 xg for 15 minutes. The supernatant was decanted off. The inverted tubes were standed on absorbent papers for 10 minutes and then trapped gently and blotted the rims to remove all residual droplets. The retaining precipitate of each vial was then counted for 1 minute in a gamma-counter.

Testosterone(T) assay

All of steroid hormones had a low molecular weight and they were prepared chemically material of virtually one hundreds percent purity (Work and Work, 1978). The non-immunogenic steroid hormones, called hapten, most were widely used for conjugation with the other proteins as albumin to induce antibody production. The site of linkage between the hapten and the protein must be done to a specific part of the steroid to achieve sufficient specificity of resulting antiserum, where there is a family of chemically related compounds with highly different biologic activity. The goal was that the binding site of the steroid hormones to antibody coincided with the part of the biologically active site (Thorell and Larson, 1978).

Testosterone determination was established in the different procedures on sample preparation (extracted and unextracted serum) and antibody production from distinguished testosterone conjugation. The assay of testosterone had a less of species specificity. Therefore, we could evaluate the hormonal levels in human (Rosenfield, Jones and Fang, 1977; Ketupanya, 1978), rhesus monkey (Goodman et al., 1974; Gilbeau et al., 1984), cynomolgus monkey (Meusy-Dessolle and Dang, 1985; Weinbauer et al., 1989) and rat (Cicero et al., 1975; Bartlett et al., 1989) by using the similar reagents. In this assay testosterone of cynomolgus monkey competed with (1,2,6,7-^{3}H) testosterone (purchased from Amersham, England) to mouse antibody to testosterone-3CMO-BSA (obtained from WHO RIA Reagent Programme) and followed WHO method.

Reagents preparation :

1. Testosterone antiserum

Testosterone antibody was produced in mouse by monoclonal antibody technique in response to testosterone-3CMO-BSA conjugate. It was provided in lyophilized form. This freeze dried antiserum was stable for several years if stored at 4 ^{o}C. The content of one bottle of antiserum was reconstituted with 10 ml buffer S before use with a final dilution in 1 : 210,000.

2. Testosterone tracer

Stock solution (1,2,6,7-^{3}H) testosterone that was provided in the concentration of 9.25 MBq(250 uCi) was transferred to a volumetric flask and filled up with toluene : ethanol (9:1) to 25 ml. This stock solution with concentration of 370 KBq (10 uCi/ml),

was stored in a dark bottle at -20 °C.

Working solution : One hundred fifty microliters of stock solution was transferred into a bottle and evaporated the solvent. Redissolved the remaining residue with 15 ml buffer S. This solution contained 3.7 KBq/ml (100 nCi/ml).

3. Testosterone standard

Carefully transferred 3 x 100 ul the stock solution (in ethanol) at a concentration of 220 nmol/l to the bottles provided. These aliquots were stored at 4 °C until needed. When required, 10 ml buffer S was added to 100 ul of ethanolic solution and heated to 40 °C in the water bath for 30 minutes. After that, the solution was mixed vigorously and allowed to cool to 4 °C before use. This solution (solution B) contained testosterone as a concentration of 2.2 nmol/l or 2,200 fmol/ml. The solution B was diluted in a serial set to obtain the following dose levels of the standard : 1100, 550, 275,138, 69, 34 and 17 fmol/500ul.

4. Recovery tracer

Recovery tracer was prepared for monitoring the recovery of extraction. One hundred microliters of stocking tracer was transferred to a vial, evaporated the solvent and redissolved in 1.0 ml buffer S.

5. Sample preparation

The sample was extracted with diethyl ether before hormonal assay. Basal circulating levels of steroid hormones were in

the low picogram or even femtogram range. The advantages of an extraction were concentration of the endogenous ligand from other materials, improvement of specificity, and avoidance of damage to endogenous ligand or tracer with potentially damaging enzymes in the sample (Chard, 1978).

5.1 Extraction of unknown serum and quality control

Ten microliters of unknown serum and quality control were aliquoted into cone tubes and reconstituted with 5 ml of fresh diethyl ether. Each tube was mixed by vortex mixer for 1 minute and allowed ether to settle. Then, tubes were dipped in 95 % ethanol containing chips of dry ice. The aqueous layer was freezed and the above ether layer was decanted to the assay tube. The ether was evaporated to dryness in a dri-block heater. Therefore, the remaining residual was redissolved with 500 ul buffer S and left 5-10 minutes then vortex mixed again. This solution was ready to be used for assay.

5.2 Extraction of recovery

Ten microliters of pool serum and 10 ul of recovery tracer were pipetted into cone tubes in duplicate, mixed and allowed the tracer to equilibrate with the serum binding proteins about 30-60 minutes at room temperature. Then the mixing reagent was extracted with diethyl ether the same as the unknown serum and decanted ether layer to the counting vial. After dryness of ether, the extract was reconstituted with 500 ul of buffer S and added scintillation fluid. Each vial was counted in beta-counter for 5 minutes. Recovery of extraction calculated by dividing the sample

recovery counts by the recovery total counts (10 ul recovery tracer and 500 ul buffer S).

Assay procedure :

The various reagents were added to yield a final volume of 900 ul the extracted unknown sample and quality control and serial dilution of standard in 500 ul of buffer S in assay tubes were added 100 ul antiserum, except NSB tubes were performed with 600 ul buffer S. All tubes were added 100 ul testosterone tracer. The reaction mixture was agitated in vortex mixer, incubated at 4 ^{o}C for 18-24 hours. The separation of antibody-bound hormone was performed in an ice bath by addition of 200 ul cold charcoal suspension which stirred continuously with a magnetic stirrer. Each tube was mixed, incubated for 15 minutes at 4 ^{o}C, then centrifuged at 1500 xg for 15 minutes. The supernatant was decanted to counting vial and reconstituted with scintillation fluid then counted in beta-counter for 5 minutes in each after allowing the vials to stand at room temperature for at least 1 hour.

Estradiol(E_2) assay

Estradiol determination in the assay was performed by two-time extraction of serum. Since estradiol levels in male monkey were very low in the range 40-100 pg/ml (Meusy-Dessolle and Dang, 1985), the two-time extraction could concentrate and recover much more hormone from sample (Figure 13). Many reports (Meusy-Dessolle and Dang, 1985; Tangpraprutigul, Cholvanich and Varavudhi, 1987) established the measurement of E_2 concentration in cynomolgus monkey which competed with E_2 tracer to rabbit antiserum to estradiol-6-

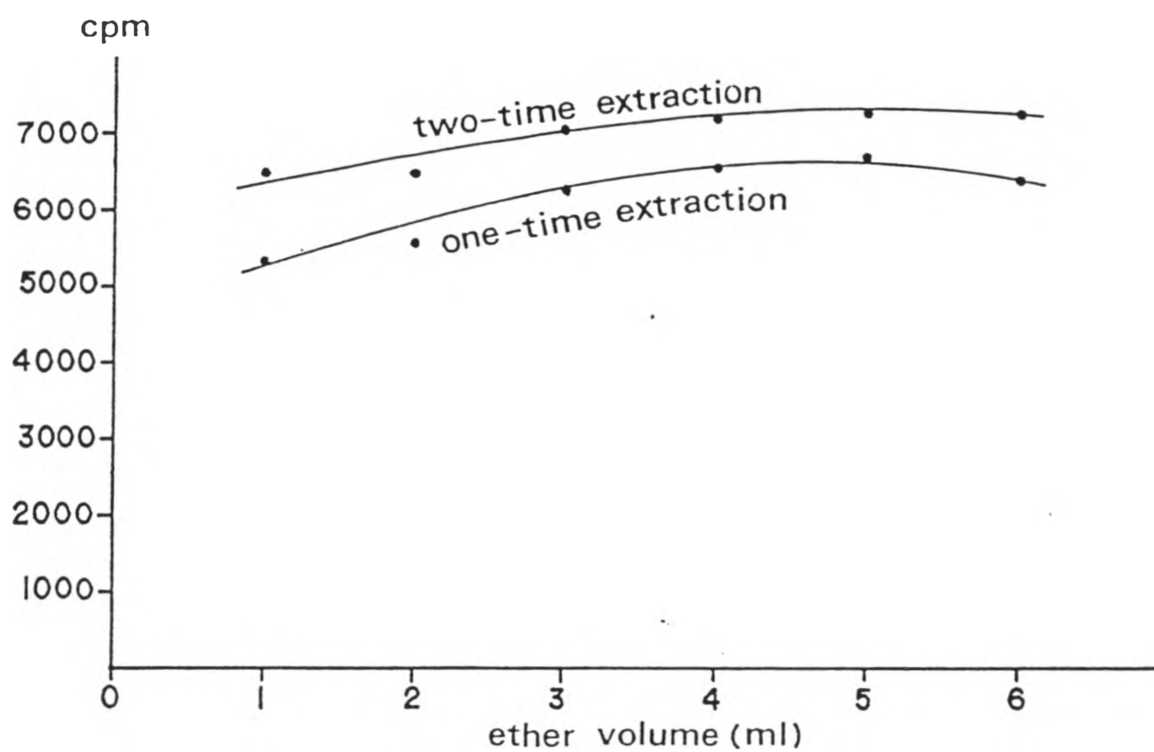

Figure 13 Checking for the maximum extraction of 500 ul pooled monkey serum mixed with 10 ul ^{3}H-estradiol (10,000 cpm) by various volumes of diethyl ether.

CMO-BSA. This assay followed WHO(1990) method.

Reagents preparation :

1. Estradiol antiserum

Estradiol antiserum was generated in the rabbit to estradiol-6-CMO-BSA. Each bottle was provided in lyophilized form and contained enough antiserum for 100 tests. It was redissolved with 10 ml buffer S for 5-10 minutes and mixed again before use. The final concentration was 1:210,000.

2. Estradiol tracer

The stock solution (concentration 370 KBq or 10 uCi/ml) was prepared from estradiol tracer [(2,4,6,7-^{3}H) estradiol] in amounts of 9.25 MBq (250uCi) by mixing with 25 ml of toluene : ethanol(9:1). Working solution was prepared by evaporation of 150 ul stock solution and redissolved in 15 ml buffer S for 30 minutes. The final concentration contained 3.7 KBq/ml (100 nCi/ml).

3. Estradiol standard

One hundred microliters aliquot of stock solution (150 nmol/l) was added 10 ml buffer S and heated at 40 ^{o}C for 30 minutes. The solution was allowed to cool to 4 ^{o}C and mixed vigorously before use. This solution contained 1.5 nmol/l or 1,500 fmol/ml E_2 and was stable at 4 ^{o}C for 2-3 weeks. Seven serial dilutions of standard E_2 as 750, 375, 187, 93, 46, 23 and 11 fmol/500 ul were prepared.

4. Recovery tracer

Recovery tracer was prepared for monitoring the recovery of extraction. One hundred microliters of stocking tracer was transferred to the vial, evaporated the solvent and redissolved in 1 ml buffer S.

5. Sample preparation

5.1 Extraction of unknown serum and quality control (two-time extraction)

Five hundred microliters of unknown serum and quality control were pipetted into cone tubes, in duplicate, and reconstituted with 5 ml fresh diethyl ether. Each tube was mixed by vortex mixer for 1 minute and allowed ether to settle. Then, all tubes were dipped in 95 % ethanol containing chips of dry ice. The aqueuos layer was freezed and the above ether layer was decanted to the assay tube. Moreover, samples were repeated extraction with 5 ml diethyl ether and poured over the first one to assay tube the ether was evaporated to dryness in a dri-block heater. Therefore, the remaining residue was redissolved with 500 ml buffer S and left for 5-10 minutes then vortex mix again. This solution was ready to be used for assay.

5.2 Extraction of recovery

Five hundreds of pool serum and 10 ul of recovery tracer were pipetted at once into cone tubes, mixed and allowed the tracer to equilibrate about 30-60 minutes at room temperature. Then the mixing reagent was extracted in the same procedure as the unknown serum extraction and decanted ether layer

to a counting vial, evaporated to dryness. The extract was reconstituted with 500 ul buffer S and added scintillation fluid. Each vial was counted in beta-counter for 5 minutes. Calculation of recovery of extraction was the same as testosterone procedure.

Assay procedure :

The various reagents were mixed to yield a final volume of 900 ul. The extracted unknown sample and quality control and seven serial dilutions of standard in 500 ul of buffer S were added with 100 ul antiserum, except NSB tubes were performed with 600 ul buffer S. All tubes were added 100 ul estradiol working tracer. The reaction mixture was agitated in vortex mixer, incubated at 4 ^{o}C for 18-24 hours. In an ice bath, 200 ul cold charcoal suspension which stirred continuously with a magnetic stirrer was added, mixed, incubated for 15 minutes at 4 ^{o}C and then centrifuged at 1500 xg for 15 minutes. The supernatant was decanted to counting vial, reconstituted with scintillation fluid 5 ml and counted in beta-counter for 5 minutes after incubation in the dark room at least 1 hour.

Cortisol assay

Cortisol concentration was high in the range 12-14.8 ug/dl in man (Tolis, Hickey and Guyda, 1975; Taylor, Dluhy and William, 1983). Therefore the determination of hormonal concentration did not consider to extract the sample from the interfering substances present in serum. The assay procedure which followed WHO(1990) method was a simple non-extraction method. Cortisol associated in virtually instantaneous with cortisol-binding globulin (CBG). At

37 ^{o}C the rate of dissociation was also rapid (half life 10 seconds) so that only a short incubation time (5-15 minutes) was needed (Slaunwhite and Sandberg,1970). Then, the assay was performed with dissociation of cortisol from CBG of which the denature was made at 60 ^{o}C for 30 minutes.

Reagents preparation :

1. Cortisol antiserum

The antiserum was rabbit antiserum which raised against cortisol-21-hemisuccinate-BSA. It was provide in lyophilized form and contained enough antiserum for 100 tests. Ten microliters buffer S was added to each bottle. After reconstitution, the solution was allowed to stand 5-10 minutes and mixed again before use.

2. Cortisol tracer

The stock solution of (1,2,6,7-^{3}H) cortisol (370 KBq or 10 uCi) was prepared by diluting tracer solution in amounts of 9.25 MBq(250 uCi) with 25 ml toluene : ethanol(9:1 v/v)

Working solution was prepared by transferring 150 ul stock solution into a bottle and evaporating the solvent. After dryness, 15 ml buffer S was added and redissolved for 30 minutes. This working tracer solution was sufficient for one assay of 100 tubes and contained 3.7 KBq/ml (100 nCi/ml).

3. Cortisol standard

Cortisol standard was provided in ethanolic solution at a concentration of 6.0 umol/l for one ampoule. Ethanolic solution

was transferred 3 x 100 ul to each bottle and stored at 4 ^{o}C until needed. When required, 10 ml buffer S was added and heated to 40 ^{o}C for 30 minutes. The solution was mixed vigorously and allowed to cool to 4 ^{o}C before use. It contained 60 nmol/l or 60 pmol/ml cortisol and was stable at 4 ^{o}C for at least 2-3 weeks. Six serial dilutions (1:1) was prepared as 6000, 3000, 1500, 750, 375 and 187 fmol/100 ul.

4. Sample preparation

From a simple non-extraction method, 15 ul of unknown serum and quality control was mixed with 1 ml distilled water. The mixing reagent was incubated at 60 ^{o}C for 30 minutes. After incubation, the solution was allowed to cool down and vortex mixed well before transferring each 100 ul aliquot to duplicate assay tubes.

Assay procedure :

One hundreds microliters of unknown serum and quality control which was prepared by a simple non-extraction method and 100 ul of six serial standards were pipetted in the assay tubes, in duplicate. Then, 100 ul antiserum and 400 ul buffer S was added to each tube, except NSB tubes were performed with 600 ul buffer S. All tubes were added 100 ul cortisol working tracer and vortex mix. The assay mixer was incubated at 4 ^{o}C for 18-24 hours. In ice bath, 200 ul of cold charcoal suspension which stirred continuously was mixed and incubated at 4 ^{o}C for 30-35 minutes. The bound form was separated by centrifugation at 1500 xg for 15 minutes. The supernatant was quickly decanted to a counting vial and

reconstituted with 5 ml scintillation fluid. The mixing reagent was left at least 1 hour before counting in beta-counter.

Statistical Analysis

The results are expressed as the mean ± SE. Student's t test was used to determine the differences between pairs of means. With three or more means, analysis of variance was used first. The observed significances were then confirmed with LSD test. p values of <0.05 were considered significant.

Table 3 Validations* of the radioimmunoassay for steroid hormones (estradiol-17B, testosterone and cortisol).

	estradiol 17-B	testosterone	cortisol
Recovery of extraction (mean ± SE)	80.49± 1.18	82.97 ± 1.64	-
Inter-assay precision (%CV)	8.7 for 24.7 pg/ml	10.3 for 8.9 ng/ml	10.1 for 237.9 ng/ml
Intra-assay precision (%CV)	5.7 for 93.7 pg/ml	6.0 for 13.0 ng/ml	5.43 for 375.0 ng/ml
Accuracy	0.963	0.961	0.976
Sensitivity	2.17 pg/ml	0.29 ng/ml	9.65 ng/ml

* The cross reaction of the antiserum with the other substances were tested by WHO Matched Reagent Programme (1986).

Table 4 Validations* of the radioimmunoassay for protein hormones (prolactin and thyrotropin) and thyroxin.

	prolactin	thyrotropin	thyroxin
Inter-assay precision (%CV)	9.2 for 1677.1 mIU/L	4.7 for 15.8 mIU/L	10.3 for 17.5 ug/dl
	6.0 for 141.4 mIU/L	9.0 for 3.6 mIU/L	6.9 for 5.2 ug/dl
	7.7 for 99.6 mIU/L	13.3 for 2.7 mIU/L	11.0 for 1.8 ug/dl
Intra-assay precision (%CV)	9.2 for 1946.5 mIU/L	5.6 for 18.2 mIU/L	8.7 for 23.6 ug/dl
	4.6 for 228.0 mIU/L	10.6 for 3.1 mIU/L	3.8 for 11.4 ug/dl
	7.2 for 157.7 mIU/L	10.8 for 2.3 mIU/L	7.2 for 3.9 ug/dl
Accuracy	0.992	0.997	0.993
Sensitivity	14.83 mIU/L	0.48 mIU/L	0.5 ug/dl

* The cross reaction of the antiserum with the other substances were tested by Diagnostic Products Corporation, LA (1992) for PRL and T_4 and by INCSTAR Corporation, USA (1990) for TSH.