CHAPTER III

MATERIALS AND METHODS

MATERIALS

- ANIMALS

Seventeen adult female cynomolgus monkeys (Macaca fascicularis (5-10 years old) with regular menstrual cycles, and 1 adult vasectomized male (13 years old) frcm the primate breeding colony of the Biology Deparment, Chulalongkorn University were used. They were caged individually under environmental temperature. The colony was exposed to natural light and additional fluorescent light were also provided daily (0600-1800 hr). They were fed daily with monkey chow (Pokphand Animal Feed Co.,Ltd., Thailand) in the morning and supplemented with fresh banana, sweet potatoes, cucumber, corn, etc, in the afternoon. Chicken boiled eggs and multi-vitamin syrup "nutroplex" (Great Eastern Co.,Ltd., USA.) were also given to those subjected blood samplings during experiment.

- CHEMICAL REAGENT AND INSTRUMENT See appendix.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

METHODS

Female monkeys with regularity of the menstrual cycle during the last 2 consecutive cycles, 33±5 days, were selected from 50 adult female monkeys in the colony. Selected monkeys were divided into 2 major sub-groups for different dosages of morphine administration: i) low and moderate doses (0.1-0.8 mg/kg /day) and ii) high doses (1.6 & 3.2 mg/kg/day).

Several evidences from the primate experimental studies showed the single dose of intravenous 0.04-1.8 mg/kg morphine

injection was given for hormonal study in rhesus monkey (Gold, Redmond and Donabedian,1979, Ferin et al.,1982, Gilbeau et al., 1985). The doses of intramuscular injection were 0.03-3.0 mg/kg for behavioual experiment in squirrel (Brady and Barrett, 1986, Craft and Dykstra,1990). Additional study on self-injection in baboons found the doses of morphine administration were 0.01-1.00 mg/kg/day (Lukas et al.,1984).

In this study, it was estimated that doses of morphine given in the second group would be sufficient to induce interruption of the menstrual cycle in most monkeys and could induced steady state of metabolic turnover of morphine during prolong period of treatment. Serum levels of reproductive hormones(E_2 and P), stress hormones (cortisol and PRL) and metabolic turnover of morphine were measured in both groups. Observation was also made on patterns of sexual interaction of each female exposed to an adult vasectomized male was also followed during pre-treatment, treatment and post-treatment cycles. Experimental schedules were summarized in table 1.

1. Low and moderate morphine doses group (n=11)

- Pre-treatment period

Five ml of venous blood samples were collected weekly in the morning (0800-0900) starting on D5 of the menstrual cycle for 2 consecutive cycles. These samples were used for RIA determination of cortisol, E_2, P and PRL. Additioal 3 ml of the blood samples were collected on D10, D14, D16, D21 and D23 for E_2 and P determination only. The turnover rate of morphine was measured twice during follicular (D8-D10) and luteal phases (D22-D25) ofthecycle. The sexual behaviour patterns of each female

monkey pairing with a vasectomized male were observed during mid-cycle (D13-D18) in all cases.

- Treatment period

The selected female monkeys were further divided into 4 sub-groups treated with different doses of morphine hydrochloride daily for 100 days. The drug was injected subcutaneously in early morning at 0800 hr around the thick region of the back of the hip as follows : 0.1(n=2), 0.2(n=3), 0.4(n=3) and 0.8(n=3) mg/kg respectively. Five ml of blood samples were collected weekly , started on each D5 of the cycle, and others 3 ml were collected on D10, D14, D16, D21 and D23 as pre-treatment cycle. The determination of the turnover rate of morphine was done on D30 and D60 of treatment period and the sexual behaviours were studied in each monkey during D13-D18 of the first cycle only.

- Post-treatment period

The post-morphine treatment effect were continuously monitored for 100 days of the withdrawal period. Blood samples (5ml) were drawn in the morning, started on D3 and D5 of the post- treatment day and followed by weekly intervals. Additional 3 ml of the blood samples were drawn from each monkey on D3, D14, D16, D21 and D23 of the cycle. The turnover rate of morphine was determined on D30 of the post-treatment period.

2. High morphine dose experimental group (n=6)

- Pre-treatment period

Five ml of blood samples were collected in the morning between 0800-0900 hr every 5 days intervals, started on D5 of the menstrual cycle for 2 consecutive cycles. These samples were used for the hormonal determination, cortisol, E_2, P and PRL.

The turnover rate of morphine were measured during follicular phase(D8-D10) and luteal phase (D22-D25) of the cycle. In addition, the sexual behaviour of each female monkey pairing with a vasectomized monkey were also observed continously during D13-D18 of the cycle.

- Treatment period

The selected female monkeys were further divided into 2 sub-groups. These monkeys were daily injected subcutaneously with morphine hydrochloride in the dose of 1.6(n=3) and 3.2(n=3) mg /kg respectively. Both groups were subjected to blood samples collection on D5 of the cycle and every 5 days intervals throughout treament period. The turnover rate of morphine was determined weekly intervals starting on D10 of the cycle. The pattern of metabolic turnover were monitored until reaching steady state. The morphine treatment periods lasted for 143 and 120 days for daily dose of 1.6 and 3.2 mg/kg morphine hydrochloride administration respectively. The sexual behaviours were studied during D13-D18 of the cycle .

- Post-treatment period

The reproductive hormonal levels, the metabolic turnover of morphine and the female sexual behaviours were also followed in this post-treatment period until the turnover rate return to pre-treatment value within 90 days in all monkey. Five ml of the blood sample were collected on day3, day5, day7 day10 after the drug withdrawal and followed by 5 days intervals until day 90 for serum determination of cortisol, E_2, P and PRL.

Table 1. Experimental schedules of 17 female monkeys injected with morphine hydrochloride.

Treatment Groups	Period of studies		
	pre-treatment	treatment	post-treatment
I. Low and moderate doses (0.1,0.2,0.4,0.8 mg/kg/day)	2 cycles	100 days	100 days
i) blood collection	D5,D10,D12,D14, D16,D19,D21,D23, D26,D33,....	D5,D10,D12,D14, D16,D19,D21,D23, D26,D33,....	D3,D5,D10,D12, D14,D16,D19,D21, D23,D26,D33,..
ii) morphine turnover rate	D8-D10, D22-D25	D30, D60	d30
iii) sexual behaviour	D13-D18	D13-D18	
II. High doses (1.6&3.2 mg/kg/day)	2 cycles	120-143 days	90 days
i) blood collection	D5,D10,D15,....	D5,D10,D15,...	d3,d5,d7,d10,d15,..
ii) morphine turnover rate	D8-D10,D22-D25	D10,D17,D24,....	d2,d4,d6,d10,d17,..
iii) sexual behaviour	D13-18	D13-18	D13-D18

D1,D2,.... = day of the cycle

d1,d2,.... = day after morphine withdrawal

Table 2. The past history of 17 monkeys on morphine treatment.

Dose of morphine mg/kg/day	Monkey No.	Sex/ approx. age,yrs.	Birth place	Parity**	Remarks
-	44	M/13	natural habitat*	-	vasectomized
0.1	601	F/10	the colony	1	
	615	F/9	the colony	-	
0.2	41	F/9	natural habitat*	1	
	61	F/10	natural habitat*	-	
	64	F/10	natural habitat*	-	
0.4	99	F/10	natural habitat*	2	
	616	F/6	the colony	-	
	800	F/8	natural habitat*	-	
0.8	71	F/10	natural habitat*	1	
	102	F/10	natural habitat*	1	
	611	F/9	the colony	-	
1.6	92	F/10	natural habitat*	4	
	93	F/10	natural habitat*	2	
	95	F/10	natural habitat*	4	
3.2	60	F/10	natural habitat*	-	
	81	F/10	natural habitat*	1	
	98	F/10	natural habitat*	2	

M = Male

F = Female

* = Obtained from Don Hoylord of Samusongkam Province

**= Recorded before experimentation

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Monkey #601

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/ = day of behavioural observation
& = day of blood collection
m = menstruation
t = day of 0.1 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal

Figure 4. The study protocol and menstrual cycle record in monkey no.601.

Monkey #615

	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	
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/ = day of behavioural observation
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m = menstruation
t = day of 0.1 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal

Figure 5. The study protocol and menstrual cycle record in monkey no.615.

Monkey #41

	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	
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/ = day of behavioural observation t = day of 0.2 mg/kg/day morphine hydrochloride administration
& = day of blood collection w = day of morphine withdrawal
m = menstruation

Figure 6. The study protocol and menstrual cycle record in monkey no.41.

Monkey #61

	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	
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/ = day of behavioural observation t = day of 0.2 mg/kg/day morphine hydrochloride administration
& = day of blood collection w = day of morphine withdrawal
m = menstruation

Figure 7. The study protocol and menstrual cycle record in monkey no.61.

Monkey #64

	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	
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/ = day of behavioural observation t = day of 0.2 mg/kg/day morphine hydrochloride administration
& = day of blood collection w = day of morphine withdrawal
m = menstruation

Figure 8. The study protocol and menstrual cycle record in monkey no.64.

Monkey #99

	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	
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/ = day of behavioural observation t = day of 0.4 mg/kg/day morphine hydrochloride administration
& = day of blood collection w = day of morphine withdrawal
m = menstruation

Figure 9. The study protocol and menstrual cycle record in monkey no.99.

Monkey #616

Month	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	Year
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/ = day of behavioural observation
& = day of blood collection
m = menstruation
t = day of 0.4 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal

Figure 10. The study protocol and menstrual cycle record in monkey no.616.

Monkey #800

Month	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	Year
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/ = day of behavioural observation
& = day of blood collection
m = menstruation
t = day of 0.4 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal

Figure 11. The study protocol and menstrual cycle record in monkey no.800.

Monkey #71

	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	
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/ = day of behavioural observation
& = day of blood collection
m = menstruation
t = day of 0.8 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal

Figure 12. The study protocol and menstrual cycle record in monkey no.71.

Monkey #102

	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	
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/ = day of behavioural observation
& = day of blood collection
m = menstruation
t = day of 0.8 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal

Figure 13. The study protocol and menstrual cycle record in monkey no.102.

Monkey #611

	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	
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/ = day of behavioural observation
& = day of blood collection
m = menstruation
t = day of 0.8 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal

Figure 14. The study protocol and menstrual cycle record in monkey no.611.

Monkey #92

	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	
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DEC				&		/	*/	/	&/	/	/			&*					&		*		m	&m	m	m	m	*m	&m	m		
JAN			&	*/	/	/	/	&/	/		*		&				*	&					&	*m	m	m	m	&m	m	m	*m	
FEB	m	&					&*						&*	w	*	&	*	&	*	&			&*				m	&m				
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/ = day of behavioural observation
* = day of turnover rate study
& = day of blood collection
t = day of 1.6 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal
m = menstruation

Figure 15. The study protocol and menstrual cycle record in monkey no.92.

Monkey #93

	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	
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/ = day of behavioural observation
• = day of turnover rate study
& = day of blood collection
t = day of 1.6 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal
m = menstruation

Figure 16. The study protocol and menstrual cycle record in monkey no.93.

Monkey #95

	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	
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/ = day of behavioural observation
• = day of turnover rate study
& = day of blood collection
t = day of 1.6 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal
m = menstruation

Figure 17. The study protocol and menstrual cycle record in monkey no.95.

Monkey #60

	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	
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/ = day of behavioural observation
• = day of turnover rate study
& = day of blood collection
t = day of 3.2 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal
m = menstruation

Figure 18. The study protocol and menstrual cycle record in monkey no.60.

Monkey #81

	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	
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/ = day of behavioural observation
• = day of turnover rate study
& = day of blood collection
t = day of 3.2 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal
m = menstruation

Figure 19. The study protocol and menstrual cycle record in monkey no.81.

Monkey #98

	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	
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/ = day of behavioural observation
• = day of turnover rate study
& = day of blood collection
t = day of 3.2 mg/kg/day morphine hydrochloride administration
w = day of morphine withdrawal
m = menstruation

Figure 20. The study protocol and menstrual cycle record in monkey no.98.

BLOOD SAMPLE COLLECTION

Blood samples (5 ml for E_2, P, PRL and cortisol, 3 ml for E_2 and P) were collected at 0800-0900 hr by femoral venupuncture in non-anesthesized monkeys. These samples were allowed to clot and serum was separated by centrifugation at 5000 g, 4 °C, 30 min. Serum was then collected and stored at 40 °C until being analyzed for hormonal assays.

BLOOD VOLUME DETERMINATION

^{51}Cr and washed cells technique, according to Silver and Febiger (1963) and the International Committee for Standardization in Hematology (1973), were used for blood volume measurement.

1. Labelling.

Approximately 3 ml of blood obtained by venupuncture added to 0.3 ml of acid citrate dextrose (ACD) solution in a sterile container. Centrifugation at approximately 1000 g for 5 minutes and subjected to supernatant was removed and discarded.

^{51}Cr-sodium chromate solution slowly added to the packed cells under continuous gentle mixing. The mixture incubated for 30 minutes at room temperature. The labelled cells washed twice in 20 ml of sterile isotonic saline. After the second wash there was less than 1% of the remaining radioactivity in the supernatant liquid. The ^{51}Cr-labelled red cells resuspended in 3.5-4 ml of isotonic saline, 5 ml of the preparation was returned to the circulation and the rest was used as a standard for radioactivity measurements.

2. Administration

A syringe and needle was filled with the labelled red cell suspension is carefully weighed, before and after injection. The weight difference represents labell red cell suspension injected. The volume injected can be the accurately calculated from density of the suspension, using the formula given below.

$$\text{Volume injected (ml)} = \frac{\text{Weight of suspension injected (g)}}{\text{Density of suspension (g/ml)}}$$

$$\text{Density of suspension} = 1.00 + \left[\frac{\text{Hb conc. of susp. (g/dl)} \times 1.097}{34}\right]$$

(This assumes that packed red cells have a Hb concentration of 34 g/ml and a density of 1.097 g/ml).

3. Preparation of standard

The well mixed red-cell suspension (500 μl) was pipetted into a 100 ml volumetric flask containing ammonia (0.4 g/ml). Then deliver 2 ml of diluted standard thus obtained into duplicated counting tubes for radioactivity measurement.

4. Sampling

At 30 minutes after the injection, 5 ml blood sample was taken from the opposite femoral veinin a heparinized tube and mixed well. Two ml of the sample was pipetted into duplicated counting tubes containing saponin for radioactivity measurements.

5. Radioactivity measurements.

The radioactivity of each sample was measured in gamma ray measurement system.

6. calculation.

The total circulating blood volume (BV) was calculated as follows.

$$BV = \frac{RCV \times 100}{Ht \times f_1}$$

BV = Total circulating blood volume

RCV = Red cell volume

Ht = Peripheral hematocrit

$f_1 = \frac{\text{Whole body hematocrit}}{\text{Peripheral hematocrit}}$ usually = 0.92

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$$RCV = SDVHv/B$$

S = Counting rate of standard (cpm per ml)

D = Dilution of diluted standard solution

V = Volume of labelled red cell suspension injected (ml)

Hv = Pack cell volume of whole blood sample corrected for trapped plasma

B = Counting rate of blood sample (cpm per ml)

METABOLIC TURNOVER RATE STUDY

Approximately 10,000 cpm of ^{3}H-morphine (calculated from the blood volume of the female monkeys 40$\pm$4.77 ml/kg and their weigh 4.5-6.7 kg) and 0.01 mg morphine hydrochloride were adjusted to 1 ml with isotonic saline solution, were injected into saphenous vein of the female monkeys. Then, 1 ml of venous blood sample was drawn from opposite side of femoral vein into syringe at 5, 15, 30, 60, 120, 240, 360 and 480 minutes after radioactive labelled morphine administration. Blood sample was immediately transferred to heparinized tube and mixed well. The plasma was separated by centrifugation at 500 g, 30 min. Pipetted 250 μl of the plasma, in duplicated, transfered to counting vials containing 5 ml scintillation fluid for radioactivity measurement. Measured the radioactivity of each sample in beta-ray measurement system, approximately one hour for 10,000 cpm of the sample which was collected at 5 minutes.

Kinetic analysis post-injected plasma morphine radioactivity was analyzed by computer (Pharmacokinetic Programme was obtained from the Department of Physiology,

Faculty of Medicine, Srinakharinwirot University) using weighted iterative nonlinear least-squares regression analysis. Data points were fitted to each of the following function:

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

These functions are consistent with standard two-compartment open pharmacokinetic model. The quantity C represents plasma morphine radioactivity at time t after the end of the injection. The coefficients A, B and the exponents α, β are hybrid quantities. The y-intercepts of the two straight lines directly provide the values of the constants A and B. The constants α and β can be directly calculated from slopes of the two straight lines (figure 21). Thus the values of this constants are determined from the half-lives of the two exponentials (Berson et al.,1953, Goodman and Noble,1968)

Since $\alpha = \ln2/t_{1/2} = 0.693/t_{1/2\alpha}$

$\beta = \ln2/t_{1/2} = 0.693/t_{1/2\beta}$

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plasma specific activity.

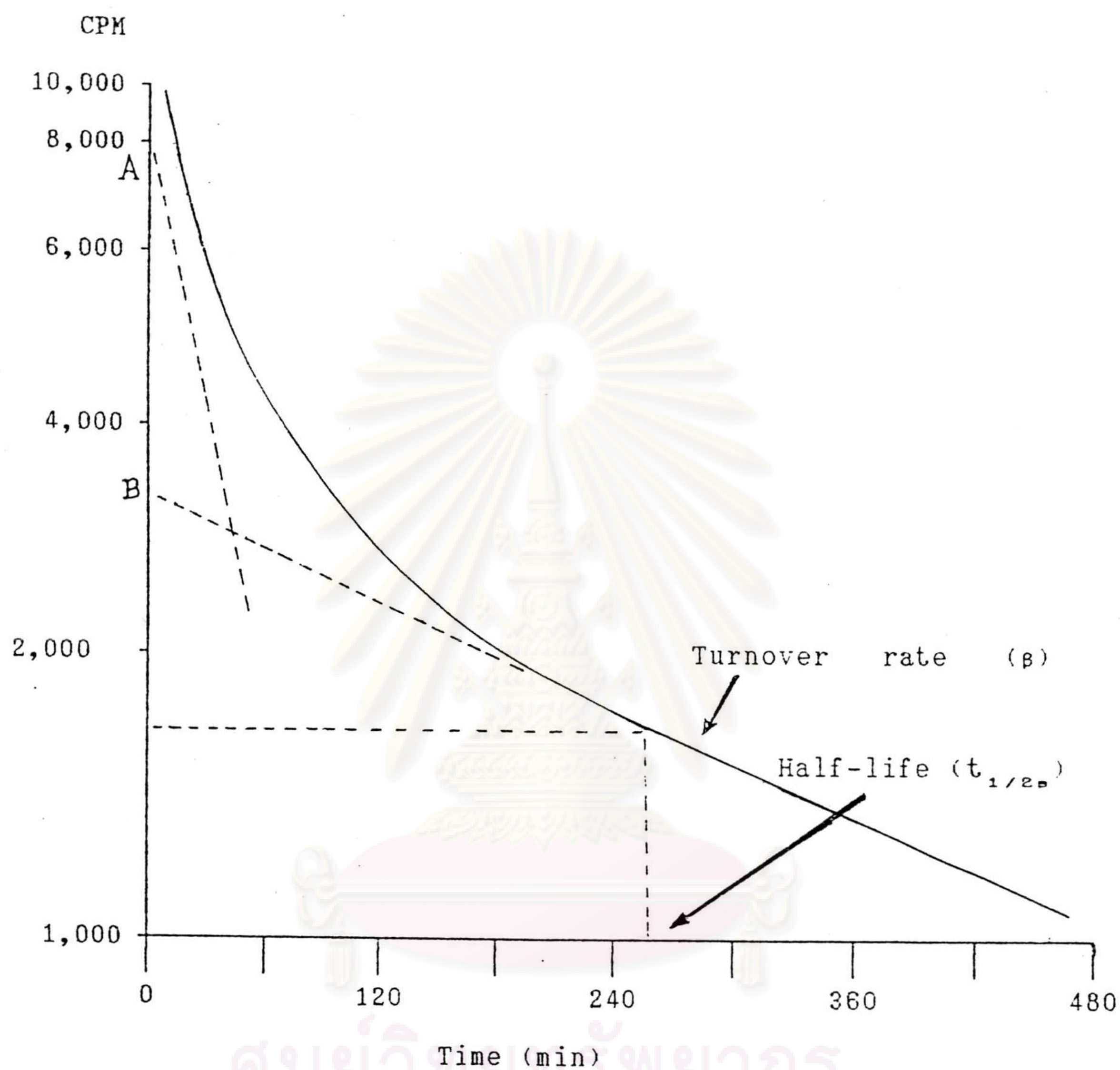

Figure 21. The kinetic of plasma morphine

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BEHAVIOURAL STUDY

Each pair of the male and female monkey was taken during the mid-cycle into a large metal grid cage (approximately 80 / 125/65 inches) which was marked one square foot each, schematically illustrated in figure 22). The cage was divided

with metal panels into three sections, the male and the female were separate, the female was in a small cage (80/24/65 inches) and the male was in another one (80/101/65 inches), both allowed into the large cage only during the experiment. The room témperature was kept at 25±1 °C with fluorescent illumination provided between 0600 and 1800. The humidity was not controlled. Daily, following the study, monkey chow, fresh fruit and vegetable were given..

1. Observation Method.

Both the female and the male were brought from their individual cages to the observation cage at least 2 days before the study. They were separated by a metal partition. The partition was removed during the observation which would tast 20 minutes for each during morning (0800-0820) and evening (1700-1720) sessions comprising one test (40 min). A number of behaviour was counted a mark, exception, time of grooming and movement were counted by time of the behaviour (minutes) and distance of movement(feet), respectively. The animals were separated again at the end of each session.

On each session of the study the observer was behind the one-way mirror and recorded their behaviour during two 20 minutes sessions, the observation was done on D13-D18 of menstrual cycle. During this experiment the panel separating female and male was removed, a clock was started and the session was begun.

2. Behaviour Recorded

This section briefly describes and operationally defines for the purpose of the observations, all behaviours were recorded here.

-Approach(AP) : onemonkey moves within arm's reach of a second monkey and remains in that position for at least 5 seconds without engaging in any other social behaviour directed at the monkey approached (Yodyingyuad,1982).

-Investigate(INV): intened visual investigation of a female's perineal sex skin. Male's face is placed within 10 cm of the female'perineum and he may sniff or touch the sex skin (Yodyingyuad,1982).

-Present(PR): female approaches male, standing slightly crouched and directing her perineal area towards him, she may look back to him then runs a short distance from the male, stops, looks back at him and presents again (Michael and Welegalla,1968).

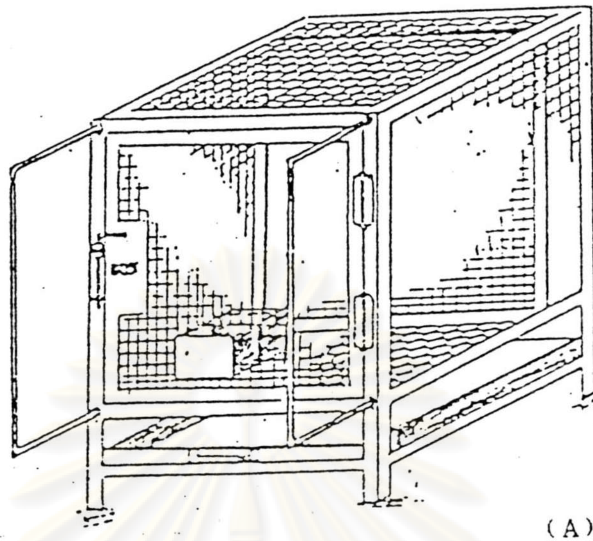
-Mount(M): male grasps the hip of female with his hands from behind and orients his genitalia towards her perineum (Phoenix,1973, Rostal and Eaton,1983).

-Invite to groom(ING): on monkey in close proximity to another monkey, a monkey lowers its head, rolls on its side or back or assumes a semi-inverted position on a perch, perhaps looking into the eyes of the other animal(Yodyingyuad,1982).

-Groom(GR): one monkey manually sifts through the fur of another occasionally moving its mouth to the skin and removing particles(Phoenix,1973, Yodyingyuad,1982).

-Yawn(Y): mouth opened wide exposing teeth, usually with head thrust back and a resultant wrinkling of skin on the back of the neck(Phoenix,1973).

-Move(MO): an animal moves to another region.



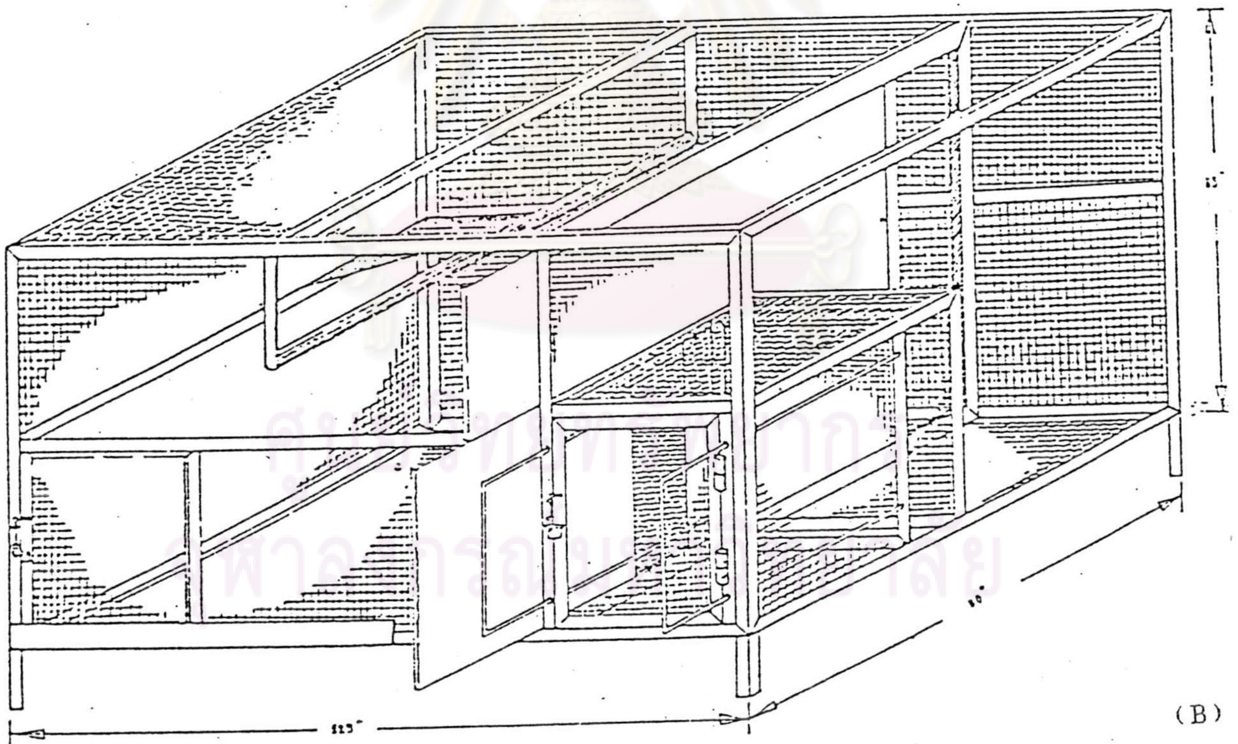


Figure 22. Individual cage (A) and observation cage (B)

Figure 23. Approach behaviour.

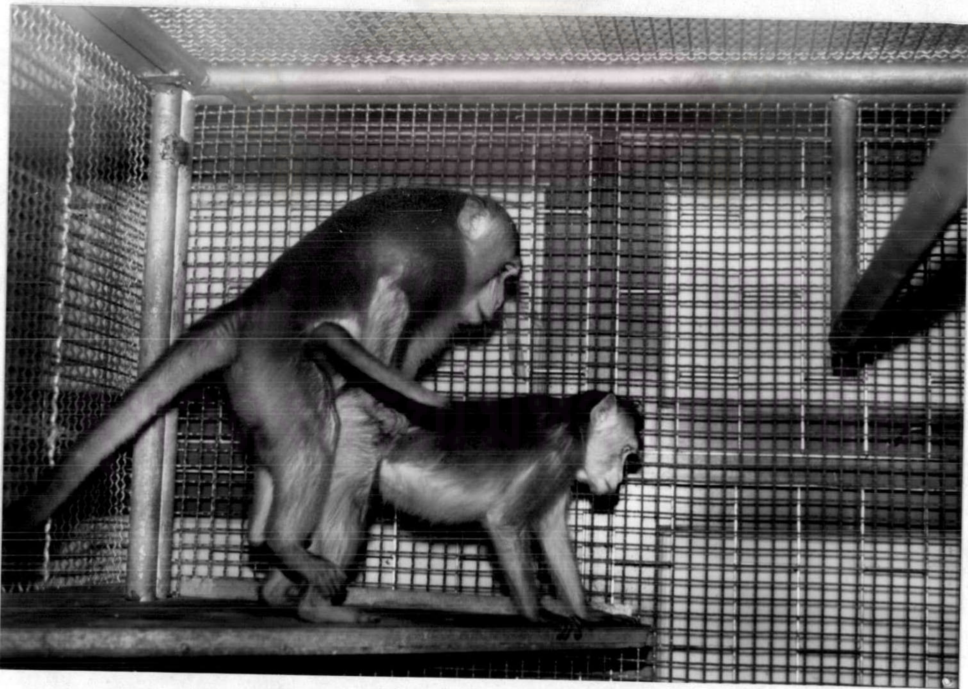


Figure 24. Mounting behaviour.

Figure 25. Grooming behaviour.

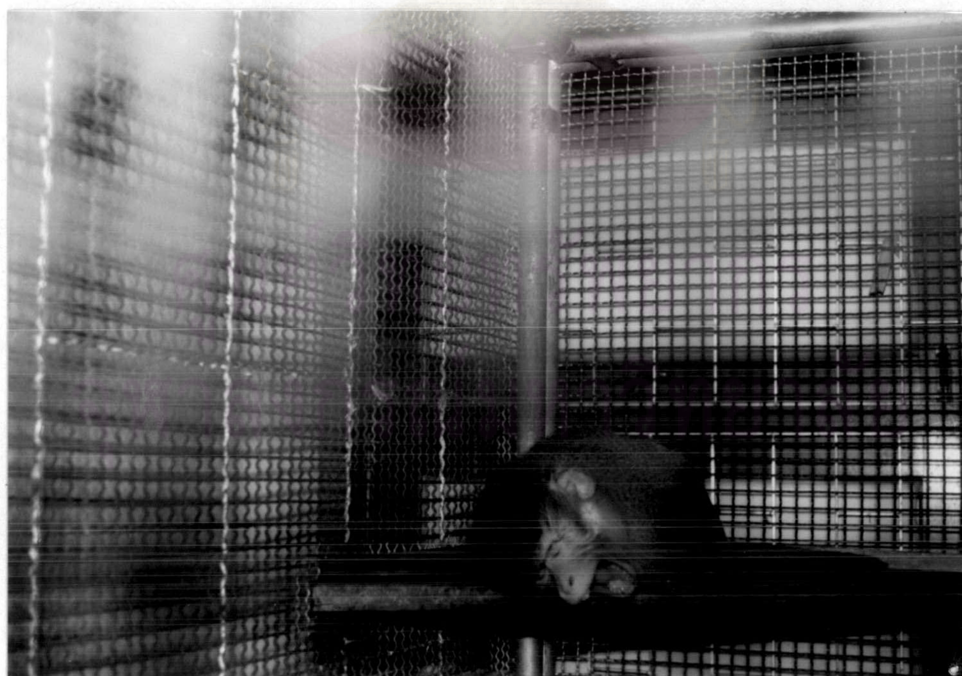


Figure 26. Yawning behaviour.

HORMONAL ANALYSIS.

I. Estradiol 17β (E_2) Assay

Estradiol reagents and assay method were provided by WHO Matched Programme (Sufi, Donaldson and Jeffcoate, 1986)

<u>Reagents and Preparation</u>

1. E_2 Standard

100 μl standard E_2 in ethanolic solution at a concentration of 160 nmol/l was mixed with 10 ml of buffer S (see appendix) and warmed to 40 °C for 30 min, then stored at 4 °C before use. This solution contain 1.6 nmol/l E_2 and stable for 2-3 weeks. Seven serial dilutions of E_2 within the range of 21-750 fmol/500 μl (tube) were prepared immediately before assay.

2. E_2 Tracer

Tritiated estradiol-17β (2,4,6,7-^{3}H-E_2, specific activity 250 μCi) was diluted to obtain 10 μCi/ml with 25 ml of tolulene: ethanol 9:1 solution and stored as the stock solution at 4 °C. Working tracer solution was preparaed by evaporation of 150 μl of the stock solution and re-dissolve in 15 ml assay buffer S. The time for resolvation of the tracer was 30 min. This solution was sufficient for one assay of 100 tubes and contained 100 nCi /ml (3.7 KBq/ml).

3. Recovery Tracer

Prepare tracer to monitor recovery by taking 100 μl stock tracer solution and reconstituting to 1 ml with buffer S after evaporating the solvent.

4. E_2 Antiserum

Anti-estradiol serum was provided in lyophilised form. One bottle of the antiserum was reconstituted with 10 ml of

assay buffer S before use. This was enough antiserum for 100 tubes.

5. Absorbent

Dextran-charcoal reagent was prepared (see appendix). Priorto use ensure the charcoal suspension must be chilled to 4 °C and well mixed.

6. Counting Solution

See appendix

Sample Preparation

Extraction method was used for preparation of the serum as following.

1. Extraction of unknown serum and quality control.

200 µlof unknown samples and quality control serums were aliquoted in duplicate tubes, extracted with 5 ml of fresh diethyl ether and vortex mixing for 1 min. Then, they were quick frozen by dipping in 95% ethanol containing chips of dry ice. The ether was decanted to assay tube and evaporated to dryness. These assay tubes were re-dissolved in buffer S for the assay procedure.

2. Extraction of recovery

200 µl of pool serum and 20 µl of recovery tracer were mixed, left and allowed to equilibrate for 30 min at room temperature. The sample was extracted with diethyl ether and then the ether was decanted to recovery vials and evaporated to dryness same as the unknown samples. These vials were also re-dissolved in 500 µl buffer S and scintillation fluid was then added. They were counted in beta counter for 5 min and calculated the percentage recovery. The recovery of extraction from assay batch to assay batch was 92.11-93.56 %.

Assay Procedure

Day 1: Each sample of the recovery, unknown and quality control was extracted and dissolved in 500 μl buffer S. 100 μl of antiserum was added, exception, NSB (non-specific binding) tubes. Then 100 μl E_2 tracer was added to each tube, mixed well and incubated at 4 °C for 18-24 hours.

Day 2: In ice bath, 200 μl of cold charcoal-dextran suspension continually mixed by magnetic stirrer was quickly added to all tubes, except Tc (total count) tubes. All tubes were vortexed and incubated for 15 min, then centrifuged at 1500 g, 4 °C for 15 min. The supernatant was quickly decanted into a counting vial added with 5 ml of scintillation fluid, capped, labelled, shaken and left for at least 1 hr then counted for 5 min each with a beta counter.

Validation of E_2

- Sensitivity

The sensitivity of this E_2 assay was 11 fmol/tube

- Precision

The coefficient variation of within assay was 5.72% for the low, 6.09% and 6.23% for intermediate and high values of quality control, respectively. For the between assay variability, coefficient variation of low, intermediate and high values of quality control were 8.65%, 12.91% and 10.42% respectively. The working range was 18.95-750 fmol/tube at the % CV = 14.12-12.67%.

- Accuracy

The correlation coefficient between the determined and added values was .95.

- Specificity

The cross reaction of antiserum with steriods likely to

be presented in the serum were tested as the following table (WHO Mathed Reagent Programme,1986).

Substance	% Cross Reaction
Estradiol	100.00
Estriol	0.80
Estrone	< 0.02
Cortisol	< 0.02
Progesterone	0.02
Testosterone	< 0.02

II. Progesterone (P) Assay.

Progesterone reagents andassaymethod were provided by WHO Matched Programme (Sufi, Donaldson and Jeffcoate,1986).

<u>Reagents and Preparation</u>

1. P Standard.

100 μl standard P in ethanolic solution at a concentration of 250 nmol/l was mixed with 10 ml of buffer S (see appendix) and warmed to 40 $^{\circ}$C for 30 min, then stored at 4 $^{\circ}$C before use. This solution contain 2.5 nmol/l P and stable for2-3 weeks. Seven serial dilutions of P within the range of19-1250 fmol/500 μl (tube) were prepared immediately before assay.

2. P Tracer

Tritiated progesterone (1,2,6,7-^{3}H-P, specific activity 250 μCi) was diluted to obtain 10 μCi/ml with 25 ml of purity tolulene solution and stored as the stock solution at 4 $^{\circ}$C. Working tracer solution was preparaed by.

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evaporation of 150 μl of the stock solution and red-issolve in 15 ml assay buffer S. The time for resolvation of the tracer was 30 min. This solution was sufficient for one assay of 100 tubes and contained 100 nCi/ml (3.7 KBq/ml).

3. Recovery Tracer

Prepared tracer to monitor recovery by taking 100 μl stock tracer solution and reconstituting to 1 ml with buffer S after evaporating the solvent.

4. P Antiserum

Anti-progesterone serum was provided in lyophilised form. One bottle of the antiserum was reconstituted with 10 ml of assay buffer S before use. This was enough antiserum for 100 tubes.

5. Absorbent

Dextran-charcoal reagent was prepared (see appendix). Prior to use ensure the charcoal suspension must be chilled to 4 °C and well mixed.

6. Counting Solution

See appendix.

Sample Preparation

Extraction method was used for preparation of the serum as following.

1. Extraction of unknown serum and quality control.

100 μl of unknown samples and quality control serums were aliquoted in duplicate tubes, extracted with 5 ml of fresh diethyl ether and vortex mixing for 1 min. Then they were quick frozen by dippingin 95% ethanol containing chips of dry ice. The ether was decanted to assay tube and evaporated to dryness. These assay tubes were re-dissolved in buffer S for the assay procedure.

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2. Extraction of recovery..

100 μl of pool serum and 20 μl of recovery tracer were mixed, left and allowed to equilibrate for 30 min at room temperature. The sample was extracted with diethyl ether and then the ether was decanted to recovery vials and evaporated to dryness same as the unknown samples. These vials were also re-dissolved in 500 μl buffer S and scintillation fluid was the added. They were counted in beta counter for 5 min and calculated the percentage recovery. The recovery of extraction from assay batch to assay batch was 91.02-93.46 %.

Assay Procedure

Day 1: Each sample of the recovery, unknown and quality control was extracted and dissolved in 500 μl buffer S. 100 μl of anitserum was added, exception, NSB tubes. Then 100 μl P tracer was added to each tube, mixed well and incubated at 4 °C for 18-24 hours.

Day 2: In ice bath, 200 μl of cold charcoal-dextran suspension continually mixed by magnetic stirrer was quickly added to all tubes, except Tc tubes. All tubes were vortexed and incubated for 15 min, then centrifuged at 1500 g, 4 °C for 15 min. The supernatant was quickly decanted into a counting vial added with 5 ml of scintillation fluid, capped, labelled, shaken and left for at least 1 hr then counted for 5 min each with a beta counter.

Validation of P

- Sensitivity

The sensitivity of this P assay was 19 fmol/tube

- Precision

The coefficient variation of within assay was 7.31% for

low the 5.93% and 5.56% for intermediate and high values of quality control, respectively. For the between assay variability, coefficient variation of low, intermediate and high values of quality control were 11.12%, 9.89% and 12.76% , respectively. The working range was 56.74-1250 fmol/tube at the % CV 13.42-9.53%.

- Accuracy

The correlation coefficient between the determined and added values was .96.

- Specificity

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

Substance	% Cross Reaction
progesterone	100.00
Cortisol	< 0.01
Testosterone	< 0.30
17α-hydroxyprogesterone	< 3.00
20α-dihydroxyprogesterone	< 3.00

III. Cortisol Assay

Cortisol reagents and assay method were provided by WHO Matched Programme (Sufi, Donaldson and Jeffcoate,1986).

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Reagents and Preparation

1. Cortisol Standard

100 μl standard cortisol in ethanolic solutionat a concentration of 6.0 nmol/l was mixed with 10 ml of buffer S (see appendix) and warmed to 40 °C for 30 min, then stored at 4 °C before use. This solution contain 60 nmol/l cortisoland stable for 2-3 weeks. Six serial dilutions of cortisol within the range of 187-6000 fmol/500μl(tube) were prepared immediately before assay.

2. Cortisol Tracer

Tritiated cortisol (1,2,6,7-^{3}H-cortisol, specific activity 250 μCi) was diluted to obtain 10 μCi/ml with 25 ml of tolulene:ethanol 9:1 solution and stored as the stock solution at 4 °C. Working tracer solution was preparaed by evaporation of 150 μl of the stock solution and redissolve in 15 ml assay buffer S. The time for resolvation of the tracer was 30 min. This solution was sufficient for one assay of 100 tubes and contained 100 nCi/ml (3.7 KBq/ml).

3. Cortisol Antiserum

Anti-cortisol serum was provided in lyophilised form. One bottle of the antiserum was reconstituted with 10 ml of assay buffer S before use. This was enough antiserum for 100 tubes.

4. Absorbent

Dextran-charcoal reagent was prepared (see appendix). Prior to use ensure the charcoal suspension must be chilled to 4 °C and well mixed.

5. Counting Solution

See appendix

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Sample Preparation

A simple non-extraction method was used for the preparation of serum by mixing 15 μl of serum into 1 ml of distilled water. The samples were incubated at 60 °C for 30 min. After leave the solution cool, vortex mixed well and transfered 100 μl aliquoats to assay tubes.

Assay Procedure.

Day1: Serum of unknown and quality control was prepared by non-extraction method and 100 μl of the solution was aliquoated into duplicate assay tubes. 100 μl of antiserum was added, exception, NSB tubes. Then 100 μl cortisol tracer was added to each tube, mixed well and incubated at 4 °C for 18-24 hours.

Day 2: In ice bath, 200 μl of cold charcoal-dextran suspension continually mixed by magnetic stirrer was quickly added to all tubes, except Tc tubes. All tubes were vortexed and incubated for 15 min, then centrifuged at 1500 g, 4 °C for 15 min. The supernatant was quickly decanted into a counting vial added with 5 ml of scintillation fluid, capped, labelled, shaken and left for at least 1 hr then counted for 5 min each with a beta counter.

Validation of cortisol

- Sensitivity

The sensitivity of this cortisol assay was 185 fmol/tube

- Precision

The coefficient variation of within assay was 7.73% for the low, 7.76% and 8.76% intermediate and high values of quality control, respectively. For the between assay variability, coefficient variation of low, intermediate and high values of

quality control were 10.61%, 8.11% and 12.95%, respectively. The working range was 298-6000 fmol/tube at the % CV = 12.51-8.19%

- Accuracy

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

- Specificity

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

Substance	% Cross Reaction
Cortisol	100.00
Cortisone	<0.10
Corticosterone	9.20
11-deoxycortisol	27.10
Progesterone	0.20
17α-hydroxyprogesterone	0.80
11α-hydroxyprogesterone	0.07
Testosterone	0.08

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IV. Prolactin (PRL) Assay

Double antibody method was applied for serum PRL levels measurement, the reagents were obtained from the Diagnostic Products Corporation (DPC), USA.

<u>Reagents and Preparation</u>

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 gentle prior to use.

2. ^{125}I-PRL

The labelled 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 standard had 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, 230, 460, 1150, 2300 and 4600 mIU/L (2nd 85/562).

4. Goat Anti-rabbit Gamma Globulin

This second antibody was dissolved in diluted PEG in saline. The precipitating solution must be kept in a cool place (2-8 °C) and should be throughly mixed before use.

PRL Assay

100 μl of blank was pipetted to the NSB and Bo tubes. 100 μl of each standards, quality control and unknown samples was aliquoated into prepared tubes. Iodinated human PRL 50 μl was added to all tubes and mixed well. 50 μl of PRL antiserum was added later to all tubes except the NSB and Tc tubes. All were vortexed and incubated for 3 hr at room temperature. Consequently, 500 μl of cold, well mixed precipitating solution was added and vortex. Separation of bound from was performed by centrifugation at 3000 g, 20 °C for 15 min. The supernatant was decanted and counted each tube for 1 min.

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Validation of PRL.

- Sensitivity

The sensitivity of this assay was 12.82 mIU/L

- Precision

The within-assay coefficients of variation were 5.87% for low value of quality control, 4.05% for intermediate value ofquality control and 8.53% for high value of quality control. The between-assay precision of low, intermediate and high values for quality control were 9.64%, 6.42% and 10.29%, respectively. The working range was 110.12-2354.40 mIU/L.

- Accuracy

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

- Specificity

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

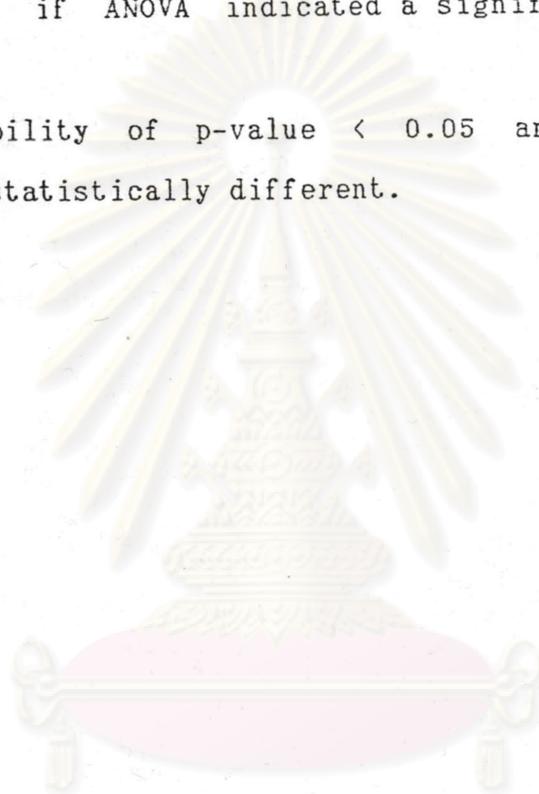
STATISTICAL ANALYSIS

Means and standard deviation were used to summarise data. Parametric statistics were used for endocrine and pharmacokinetic data, and non - parametric statistic for behaviour variables. Because of high individual varietion, the behaviour values during treatment and post-treatment periods of each monkey were compared to pre-treatment period while the values of endocrine and pharmacokinetic data were compared with the pre-treatment mean values of all 17 monkeys. In endocrine and pharmacokinetic data were used two-tail Students's t-test (unpaired) to detect difference between the mean of two samples.

Two-way analysis of variance (ANOVA) were used to detect difference among multiple samples and Turkey's test to partition sample into contrasting subsets..

Behaviour data wereused to the Mann-Whitney test (U-test) for comparison of two samples. For multiple samples, the Kruskal-Wallis one-way ANOVA was used, followed by individual Mann-Whitney test if ANOVA indicated a significant difference among groups.

A probability of p-value < 0.05 and < 0.01 were considered to be statistically different.



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