Chapter 3

Materials and Methods

3.1 Subjects

A captive population of *Amyda cartilaginea* was maintained in a turtle pond at Prayurawongsawas temple, Bangkok, Thailand under semi-natural conditions. Most of these softshell turtles were previously wild-caught and then brought alive to be liberated in the pond by merit-making people according to the Buddhist faith. The softshell turtles were adapted to the pond conditions and could perform natural habits including nesting behavior (van Dijk, 1992; Wichase Khonsue, 1993; Wachira Kitimasak, 1996). Figure 3-1 shows general appearance of the pond.

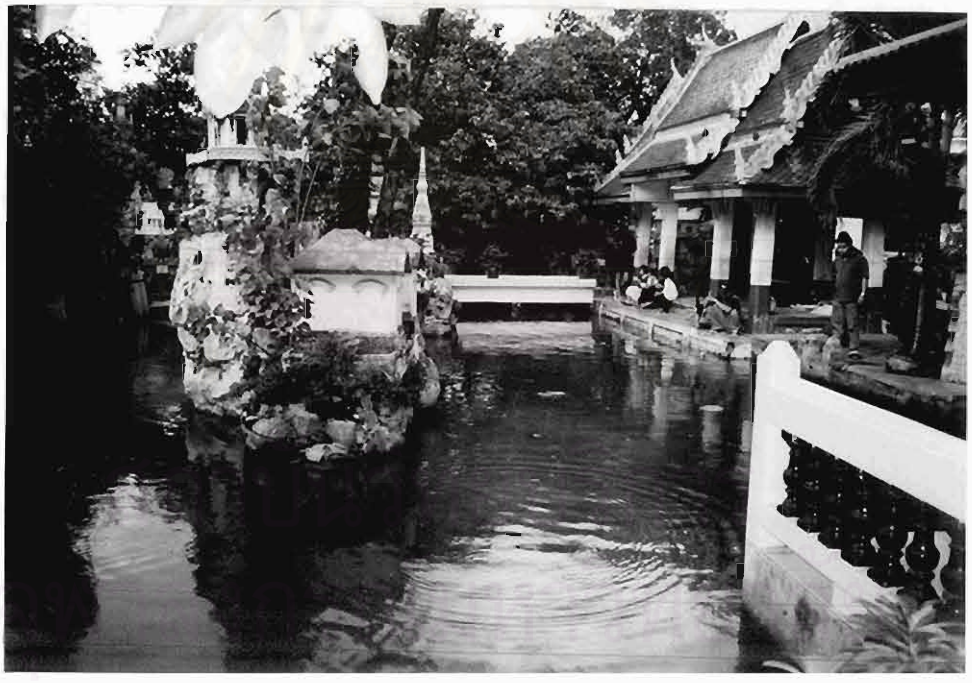

Figure 3-1 General view of turtle pond at Prayurawongsawas temple, Bangkok, Thailand.

The pond is 42 x 48 m. in size and contains a small island with nesting sand in the middle. Depth ranges from 0 m at the nesting area to a maximum depth of 3 m. The nesting area is available year-round. The softshell turtles are exposed to natural photoperiod and weather conditions. Freshwater from

the nearby Chao Phraya river is circulated through the pond every week. Mean air temperature of the Bangkok area was reported to ranged from 26.3 °C in December 1996 to 31.1 °C in May 1997. Mean relative humidity varied from 61 % in December 1996 to 76 % in September 1997. Mean daily rainfall ranged from 0 mm in December 1996 and January 1997 to 12.0 mm in September 1997. Mean daily sunshine duration ranged between 3.1 hours in July 1997 and 8.6 hours in March 1997. A monthly climatic data of Bangkok area is charted in Figure 3-2.

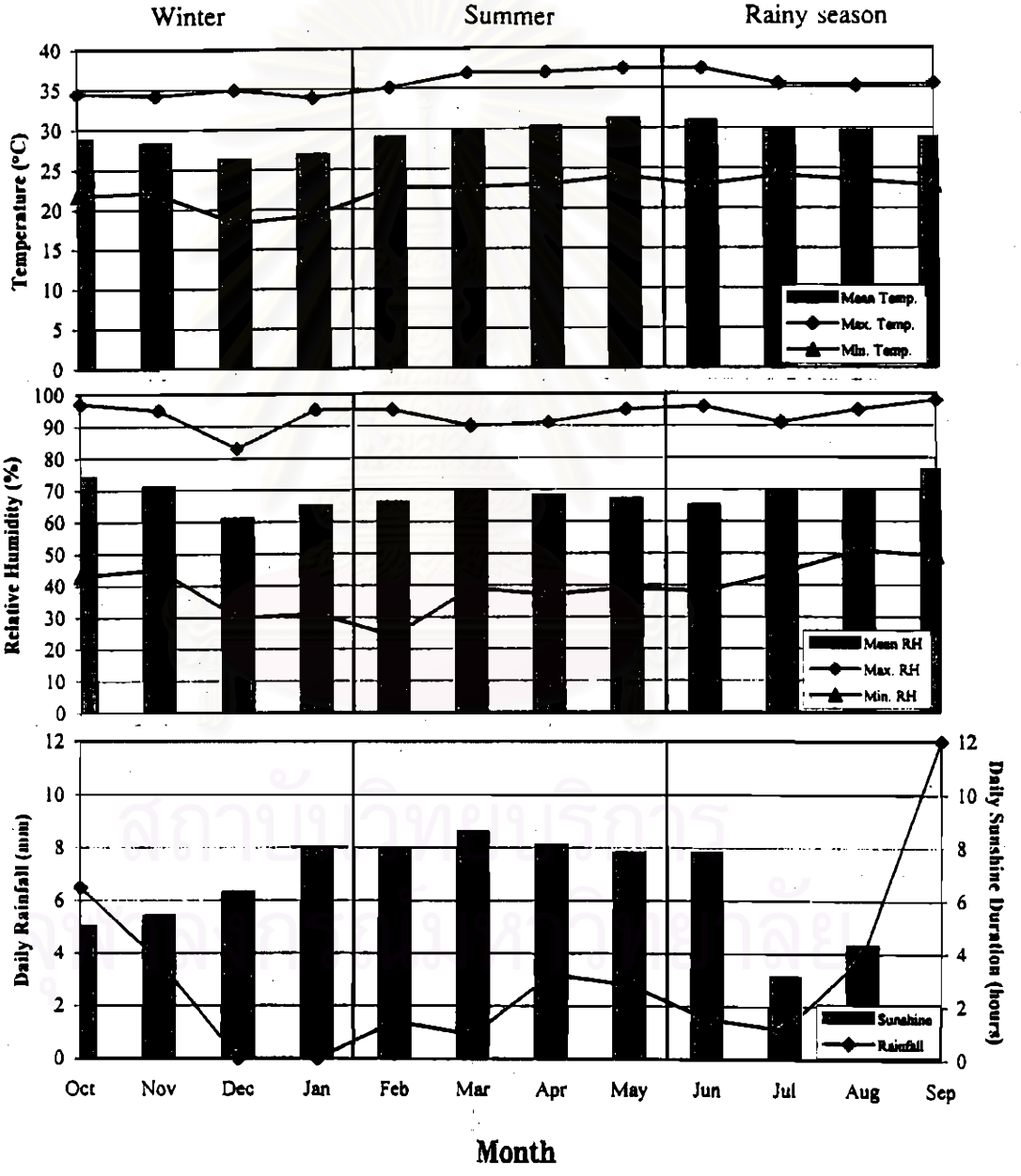

Figure 3-2 Climatic data of Bangkok Metropolis area during October 1996 to September 1997 (Meteorological Department, 1997).

* Total sunshine duration of September 1997 was not reported.

The softshell turtles can forage naturally for fish and carrion of dead turtles and are also fed with balls of fish by merit-making people.

During the one-year study period (October 1996 - September 1997), mature softshell turtles (according to Sujin Nukwan, Panu Tavarutmaneegul and Anusin Inkuan, 1995) were sampled from the pond with dip-nets during 3:00 pm - 6:00 pm. After measurements for morphological characters and blood collections, the softshell turtles were released back to the pond. Due to unfavorable water quality of the pond, the softshell turtle was hence released without marking in order to minimize incidence of infection. Sampling was carried out monthly except in October, November, December and February when samples were taken biweekly. The total number and number of each sex in each sample varied, due to softshell turtle occurrence and opportunities to catch them. The number of softshell turtles in each sample is shown in table 3-1.

Table 3-1 Number of softshell turtles in each sample from October 1996 to September 1997.

Sample	Number of Male	Number of Female	Total Number
October 1996 (1/2)	3	5	8
October 1996 (2/2)	-	5	5
November 1996 (1/2)	4	2	6
November 1996 (2/2)	6	3	9
December 1996 (1/2)	7	4	11
December 1996 (2/2)	5	4	9
January 1997	5	4	9
February 1997 (1/2)	7	3	10
February 1997 (2/2)	7	2	9
March 1997	6	3	9
April 1997	5	4	9
May 1997	5	3	8
June 1997	8	2	10
July 1997	4	-	4
August 1997	7	3	10
September 1997	1	7	8
Total	**80**	**54**	**134**

3.2 Sexual Dimorphism

The sampling softshell turtles were weighed with a commercial balance rated to 50 kg and measured for the following 18 morphological characters using a measuring tape or a vernier caliper. These parameters are illustrated in figure 3-3.

1. Carapace Length (CL): Curved maximum length of dorsal shell including posterior cartilaginous flap.
2. Carapace Width (CW): Straight maximum width of the dorsal shell including cartilaginous margin. The curved CW was abandoned to minimize error from irregular shell dome.
3. Bony Disc Length 1 (BDL1): Curved maximum length from nuchal tubercular ridge to the posterior of the dorsal bony shell.
4. Bony Disc Length 2 (BDL2): Curved maximum length of the dorsal bony shell. BDL2 represents the actual bony disc length but contains small error due to difficulty in locating the anterior end of bony disc.
5. Height (H): Maximum height when head is out.
6. Plastron Length 1 (PL1): Maximum length of the ventral bony shell from entoplastron to xiphiplastron.
7. Plastron Length 2 (PL2): Maximum length of the ventral bony shell from epiplastron to the xiphiplastron.
8. Plastron Width (PW): Width of the ventral shell measured between hyoplastron-hypoplastron conjunction including cartilaginous margin.
9. Plastron to Rear Margin of Carapace (PR): Length from end of the xiphiplastron to end of the posterior cartilaginous flap.
10. Plastron to Cloaca (PC): Length from end of the xiphiplastron to middle of cloacal opening.
11. Tailbase to Cloaca (TC): Length from tailbase to the middle of cloacal opening.
12. Cloaca to Tail Tip (CT): Length from the middle of cloacal opening to tail tip.
13. Tail Length (TL): Maximum length from the tail base to the tail tip.
14. Tail Width (TW): Maximum tail width.
15. Head Length without snout (HL): Length of head.
16. Head Length with snout (HLs): Length of head including snout.
17. Head Width (HW): Maximum width of head.
18. Head Height (HH): Maximum height of head.

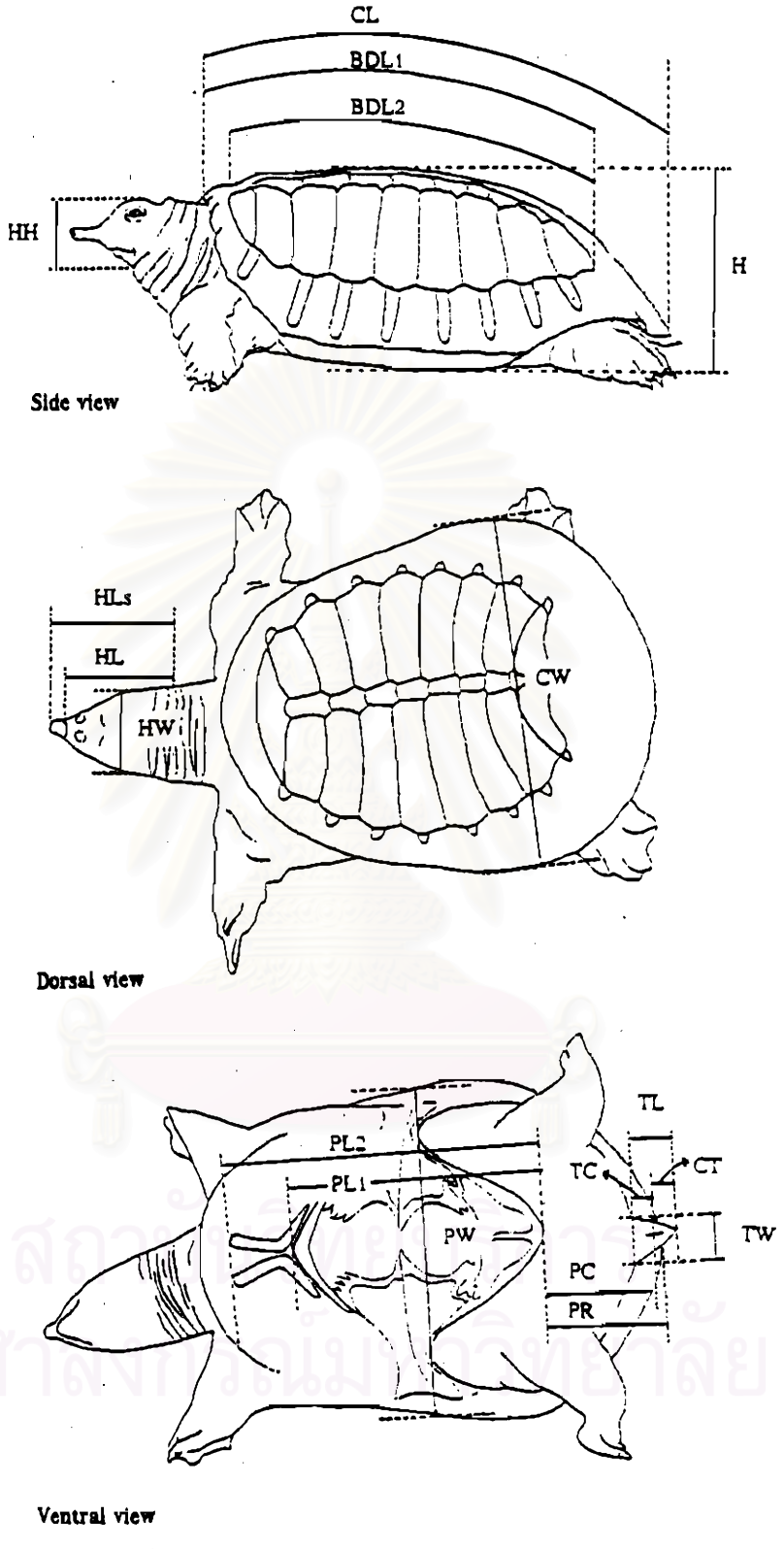

Figure 3-3 Eighteen morphological characters that were measured for the study of sexual dimorphism (side view was depicted from museum specimen: CUMZ (R) 1990-11-9-2, dorsal and ventral views were depicted from sampled specimen: BP1370997).

Eighty male and fifty-four female softshell turtles were measured during the study period. Sexual dimorphism was analyzed according to the following two procedures.

1. Mean Comparison

In order to minimize size bias, the recorded morphological characters were transformed into relative quantity to carapace length (CL). The mean relative parameters were then compared between sexes using Student's t-test.

In addition to the aforementioned parameters, recorded morphological characters were tranformed to the following parameters: tail length beyond carapace margin, body outline, and cloacal position.

Tail length beyond carapacial margin was transformed from subtraction of plastron to rear margin of carapace from addition of plastron to cloaca and cloaca to tail tip {(PC+CT)-PR}. The results were transformed into relative numbers to carapace length and compared between sexes.

Body outline i.e. dorsal view of softshell turtle body such as oval, round, etc. was determined from subtraction of carapace width by plastron width (CW-PW) and then transformed into relative number to carapace length. The result of CW-PW represented difference between the maximum width of the dorsal shell and the width of the ventral shell measured between hyoplastron-hypoplastron conjunction which was hypothesized to be relatively constant position in most individuals. Higher number of CW-PW suggested the greater in posterior portion of softshell turtle body outline.

Cloacal position was determined from division of tailbase to cloaca with tail length (TC/TL). The number represented relative position of the cloacal opening. The lower number indicated relatively anterior position while higher number indicated relatively posterior position.

In every comparisons, probability of $p \leq 0.05$ was considered to be significantly different.

2. Regression Analysis

Every morphological characters of each sex was plotted against carapace length (CL), furthermore tailbase to cloaca was also plotted against tail length. The regression analysis were performed, and regression lines were tested for significance ($p \leq 0.05$) using Analysis of Variance (ANOVA). The slopes and elevations of the regression lines between sexes were compared using Student's t-test. Significant difference was considered from probability of $p \leq 0.05$.

General calculations were performed on computer by Microsoft Excel for Windows 95 version 7.0. Statistical analysis were performed on computer by SPSS for Windows release 7.0 except the comparisons for slopes and elevations of the regression line, which were hand-calculated following Zar (1984).

3.3 Annual Reproductive Cycle

After capture, the softshell turtles were allowed to acclimatize on land for 1 - 3 hours. They were weighed and then anaesthetized with ketamine hydrochloride at a dosage of 12.5 mg/kg intramuscularly. Five to ten millilitres of blood was withdrawed from the jugular vein of each individual with 38 mm 25 gauge needles and heparinized syringes. Blood samples were stored on ice up to 5 hours until cool centrifugation at 4°C, 1100 x g. Plasma samples were separated and frozen in small aliquots of 1.4 ml at -20°C until steroids assays.

Plasma samples were assayed for levels of testosterone (T), estradiol-17β (E_2) and progesterone (P) by radioimmunoassay procedures according to WHO matched reagent programme. The WHO assay method is designed to estimate steroid levels in human serum or plasma extracts. To quantify steroids in other species, it is advised to validate the assay method beforehand (Sufi, Donaldson and Jeffcoate, 1990). The validations included parallelism check for immunological similarity and reliability of the assay (Sukanya Werawatgoompa, 1982). The reliability of RIA is assessed by four main criteria: specificity, sensitivity, precision and accuracy (Chard, 1978, Thorell and Larson, 1978 both cited in Suchinda Malaivijitnond, 1994).

Parallelism:

Parallelism is interpreted as evidence of immunological similarity or as suggesting that observed immunoreactivity is not an assay artifact (Belfe, 1987; Kieffer and Malarkey, 1978; Kyle et al., 1987 cited in Kesorn Suwanprasert, 1991).

Softshell turtle plasma containing a high level of each steroid was diluted with hormone free plasma prior to assay (preparation of hormone free plasma is described in appendix II). The dilution curve was compared to the standard curve of human steroids. The parallelism was expressed as insignificant difference between slopes of the two regression lines (Sukanya Werawatgoompa, 1982; Zar, 1982).

Specificity:

Specificity is an ability of the assay to measure one specific compound and no other. The identifiable materials with physicochemical similarity to the ligand that can interfere with the assay by reacting directly with the binder are called cross-reacting materials. The most common way to present the specificity is to compare the amount of the cross-reacting materials under study which yields 50 percent inhibition of binding (x) with the amount of standard giving the same inhibition (y), and then express the potency of the cross-reacting materials as a percentage of the standard: Percent cross-reactivity = (y / x) x 100.

The cross reaction of WHO antiserum with other substances were tested by WHO matched reagent programme (Sufi, Donaldson and Jeffcoate, 1990).

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 based on the confidence limits to the zero standard estimate. The sensitivity of an assay critically depend on the precision of the assay.

Blank test and standard were plotted together in a dose response curve of $\log_{10}$ dose against percent bound (% B/B0). The 95 % bound was determined as sensitivity of the assay.

Precision:

Precision, or reproducibility, is a measure of observed variation between repeated determination of the same sample. It is usually expressed as coefficient of variation. Intra-assay, or within assay, variation refers to precision within an individual assay. Inter-assay, or between assay, variation refers to precision from different sets of assays of the same sample. To monitor precision, the pooled samples should be set up and their concentrations 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.

Due to the limited amount of softshell turtle plasma, the pooled plasma was set up into two groups according to sex representing two different values in each assay. By the way it was found that the pooled plasma of each sex was subjected to contamination with plasma of other sex due to early miss-sexing. Thus the steroid levels in each pooled plasma did not represent the true value of each sex. It was actually valid only for monitoring the precision.

Accuracy:

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

3.3.1 Testosterone assay

To perform radioimmunoassay for testosterone of softshell turtles, testosterone of the softshell turtles was allowed to compete with (1, 2, 6, 7-^{3}H) testosterone in binding to mouse antibody to testosterone-3CMO-BSA. Dextran-charcoal was utilized for separation of free from antibody-bound hormone. The following method procedures were according to WHO matched reagent programme (Sufi, Donaldson and Jeffcoate, 1990).

Reagent preparation:

1. *Testosterone antiserum*

Testosterone antiserum was produced in mice by monoclonal antibody technique in response to testosterone-3CMO-BSA conjugate. It was provided in lyophilized form. The freeze dried antiserum is stable for several years if stored at 4°C. The content of one bottle of antiserum was reconstituted with 10

ml assay buffer solution, allowed to stand for 5-10 minutes and mixed before use with a final dilution of 1: 210,000 in assay tubes.

2. *Testosterone tracer*

Solution of (1, 2, 6, 7-^{3}H) testosterone that was provided in the concentration of 9.25 MBq (250 μCi) was transferred from sealed ampule to a 25 ml volumetric flask. The ampule was rinsed with a solution of toluene: ethanol (9:1 by volume), and the washings was added to the contents of the flask. The volume of the stock solution was made up to 25 ml with the toluene: ethanol (9:1) solution. The stock solution with concentration of 370 kBq or 10 μCi/ml was stored in a dark bottle at -20°C.

Working tracer was prepared as follows: 300 μl of the stock solution was transferred into a bottle and the solvent was allowed to evaporate. The residue was redissolved with 12 ml assay buffer solution. The bottle was allowed to stand for 30 minutes in order to resolve the tracer.

3. *Testosterone standard*

This was provided in ethanolic solution at a concentration of 220 nmol/l. An ampule and pipettes to be use were cooled to 4 °C. After the ampule was opened, aliquots of 3 or 4 x 100 μl were transferred to the 15 ml bottles. These aliquots were stored at 4°C until needed. When required, 10 ml assay buffer solution was added to the bottle and heated to 40°C in a water bath for 30 minutes. The solution was mixed vigorously and allowed to cool to room temperature before use. This solution (solution B) contained 2.2 nmol/l or 2,200 fmol/ml testosterone and was stable for 2-3 weeks when stored at 4°C. Upon assay, the solution B was serially diluted to obtain the following triplicate standard dose levels: 1100, 550, 275, 138, 69, 34 and 17 fmol/500 μl.

4. *Recovery tracer*

Recovery tracer was prepared to monitor the recovery of extraction. Three hundred microlitres of stock tracer was transferred to a vial, the solvent was allowed to evaporate and redissolved in 1.2 ml assay buffer solution.

5. *Sample preparation*

Plasma of softshell turtle was extracted with diethyl ether prior to assay. The advantages of an extraction were to concentrate endogenous ligand from

other materials, to improve specificity and to avoid damage to endogenous ligand or tracer with potentially damaging enzymes in the sample (Chard, 1978, cited in Suchinda Malaivijitnond, 1994).

5.1 *Extraction of unknown plasma and quality control*

Two aliqouts of each sample (500 µl for female, and 10 µl for male) and 3 replicates of each quality control were transferred into cone tubes and reconstituted with 5 ml fresh diethyl ether. Five millilitres of fresh diethyl ether only was added to two tubes and labelled as ether blank. Every tubes was subjected to extraction under agitation machine for 3 minutes. The ether was allowed to settle. The tubes were dipped in 95 % ethanol containing chips of dry ice. An aqueous layer froze and the upper ether layer was decanted to an assay tube. The ether was evaporated to dry in a heating block. The remaining residue was redissolved with 500 µl of assay buffer solution. The assay tubes were mixed under agitation machine for 1 minute, allowed to stand for 10 minutes then mixed again for 1 minute. This solution was then ready to be used for assay.

5.2 *Recovery of extraction*

Two aliquots of 500 µl and 10 µl of the male pooled plasma were added with 10 µl of recovery tracer in cone tubes, mixed and allowed to equilibrate with the tracer for 30-60 minutes at room temperature. The mixed aliquots were extracted with fresh diethyl ether and separated for the ether layer as well as the unknown plasma. The ether was evaporated to dry in a heating block. The remaining residue was redissolved with 500 µl of assay buffer solution, and mixed like the unknown sample. The reconstitution was decanted to counting vial. Two recovery totals were prepared by adding 10 µl of recovery tracer and 500 µl of assay buffer solution into assay tubes, then transferred to counting vials. Five millilitres of scintillation fluid was added to counting vials. After equilibration, each vial was counted in a β-counter for 5 minutes. Recovery of extraction was calculated by:

$$\text{Recovery of extraction (\%)} = \frac{\text{Sample recovery counts}}{\text{Recovery total counts}} \times 100$$

Recovery of extraction in this study ranged from 83.51 to 92.10 %. This recovery factor was merely used for monitoring efficiency of extraction in each assay. It was not applied to calculation of testosterone concentration. Hence, an underestimation for testosterone level should be remarked.

Assay procedures:

Various reagents were added to yield a final volume of 900 µl in an assay tube. Three replicates of zero antigen or maximum binding (B0) tubes were prepared by adding 500 µl of assay buffer solution in assay tubes. A 100 µl amount of antiserum to testosterone was added to the B0, extracted unknown sample, quality controls, and serial dilutions of standard in 500 µl of assay buffer solution in assay tubes. Three non-specific binding (NSB) tubes were prepared by adding 600 µl of assay buffer solution in assay tubes. Three total count (TC) tubes were prepared by adding 800 µl of assay buffer solution in assay tubes. The latter two triplicates were prepared without added antiserum. A 100 µl amount of testosterone working tracer was added to each tube, then mixed and incubated at 4°C for 18-24 hours. Contents of testosterone assay tubes are summarized in table 3-2.

Table 3-2 Summary of contents of assay tubes in testosterone assay.

	Buffer	**Standard or Sample**	**Antiserum to testosterone**	**Testosterone tracer**	*Incubate*	**Charcoal suspension**
Total count	800 µl	-	-	100 µl	*at*	-
NSB	600 µl	-	-	100 µl	*4°C*	200 µl
B0	500 µl	-	100 µl	100 µl	*18-24*	200 µl
Standard or Sample	-	500 µl	100 µl	100 µl	*hours*	200 µl

Separation of antibody-bound hormone was performed in an ice bath by addition of 200 µl of cold charcoal suspension which was stirred continuously with magnetic stirrer except for the total count tubes. Each tube was mixed under agitation machine for 1 minute and allowed to stand for 30 minutes at 4°C, then centrifuged at 1,100 x g, 4°C for 15 minutes. The supernatant was decanted into a counting vial. Five millilitres of scintillation fluid was added to each vial, and allowed to equilibrate for 60 minutes. Each vial was counted in a β-counter for 5 minutes.

A dose response curve was plotted using $\log_{10}$ dose against standard bound-NSB (counts per minute), and regression analysis was performed. Result for each sample (fmol/tube) was read from the regression line of the standard curve. This result was transformed to concentration per millilitre (fmol/ml) according volume of the sample. It was then multiplied by molecular weight of testosterone

(288.43) and divided by 10^6 in order to express the concentration of testosterone as nanogram per millilitre (ng/ml).

Validation of assay:

1. *Parallelism*

The high levels of softshell turtle testosterone were paralleled against the standard curve of human testosterone as shown in figure 3-4. There was no significant difference between the slopes of these two regression lines.

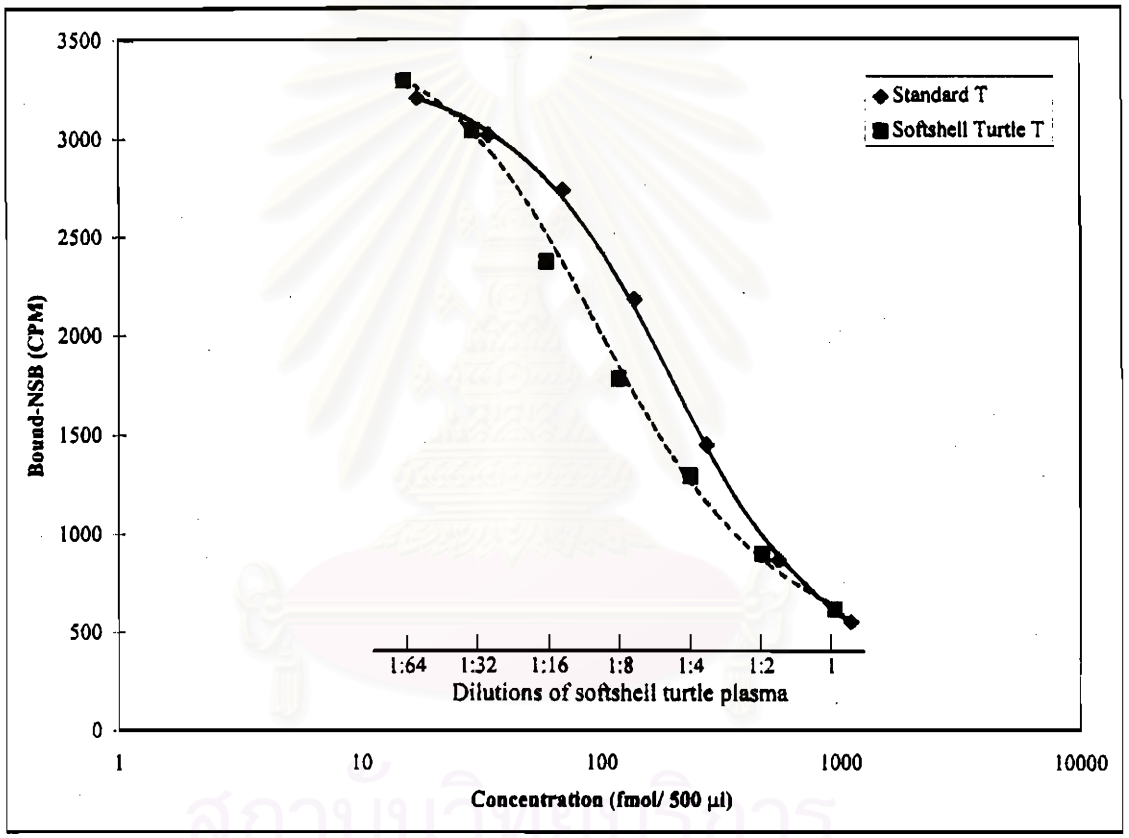

Figure 3-4 Parallelism of immunoreactivities of human testosterone standard and serial dilutions of softshell turtle plasma.

2. *Specificity*

The cross reactions of steroids likely to be present in plasma were tested by WHO matched reagent programme as listed below (Sufi, Donaldson, Jeffcoate, 1990).

- Testosterone %Cross reaction = 100.0
- 5α Dihydrotestosterone %Cross reaction = 1.3
- Δ 4-Androstenedione %Cross reaction = 3.5

- 5α-Androstanediol %Cross reaction = 1.3
- Androstane 3α diol %Cross reaction = 0.7
- Androstane 3β diol %Cross reaction = 1.8

3. *Sensitivity*

The sensitivity of testosterone assay was 3.31 pg/ml.

4. *Precision*

The intra-assay and inter-assay variation of testosterone assay are presented as coefficients of variation in table 3-3.

Table 3-3 Precision of testosterone assay.

Quality Control	Mean (fmol / 10 µl)	Coefficient of Variation	
		Intra-assay (n=10)	Inter-assay (n=30)
Males	659.08 ± 72.48	5.34 - 8.81	11.00
Females	478.32 ± 70.27	4.50 - 5.19	14.69

5. *Accuracy*

The Pearson's correlation coefficient between the determined and added values of testosterone was 0.999 ($p < 0.01$).

3.3.2 Estradiol-17β assay

To perform radioimmunoassay for estradiol of softshell turtles, estradiol of softshell turtles was allowed to compete with (2, 4, 6, 7-^{3}H) estradiol in binding to mouse antibody to estradiol-6CMO-BSA. Dextran-charcoal was utilized for separation of free hormone from antibody-bound hormone. The following method procedures were followed the WHO matched reagent programme (Sufi, Donaldson and Jeffcoate, 1990).

Reagent preparation:

1. *Estradiol antiserum*

Estradiol antiserum was produced in mice by monoclonal antibody technique in response to estradiol-6CMO-BSA conjugate. It was provided in lyophilized form. The freeze dried antiserum is stable for several years if stored at 4°C. The content of one bottle of antiserum was reconstituted with 10 ml assay

buffer solution, allowed to stand for 5-10 minutes and mixed before use with a final dilution of 1: 210,000 in assay tubes.

2. *Estradiol tracer*

Solution of (2, 4, 6, 7-^{3}H) estradiol that was provided in the concentration of 9.25 MBq (250 μCi) was transferred from sealed ampule to a 25 ml volumetric flask. The ampule was rinsed with a solution of toluene: ethanol (9:1 by volume), and the washings was added to the contents of the flask. The volume of the stock solution was made up to 25 ml with the toluene: ethanol (9:1) solution. The stock solution with concentration of 370 kBq or 10 μCi/ml was stored in a dark bottle at -20°C.

Working tracer was prepared as follows: 400 μl of the stock solution was transferred into a bottle and the solvent was allowed to evaporate. The residue was redissolved with 12 ml assay buffer solution. The bottle was allowed to stand for 30 minutes in order for resolvation of the tracer.

3. *Estradiol standard*

This was provided in ethanolic solution at a concentration of 150 nmol/l. An ampule and pipettes to be use were cooled to 4 °C. After the ampule was opened, aliquots of 3 or 4 x 100 μl were transferred to the 15 ml bottles. These aliquots were stored at 4°C until needed. When required, 10 ml assay buffer solution was added to the bottle and heated to 40°C in a water bath for 30 minutes. The solution was allowed to cool to 4°C and mixed vigorously before use. This solution (solution B) contained 1.5 nmol/l or 1,500 fmol/ml estradiol and was stable for 2-3 weeks when stored at 4°C. Upon assay, the solution B was serially diluted to obtain the following triplicate standard dose levels: 750, 375, 188, 94, 47, 23 and 12 fmol/500 μl.

4. *Recovery tracer*

Recovery tracer was prepared to monitor the recovery of extraction. Four hundred microlitres of stock tracer was transferred to a vial, the solvent was allowed to evaporate and redissolved in 1.2 ml assay buffer solution.

5. *Sample preparation*

Plasma of softshell turtles was double extracted with diethyl ether prior to assay. The advantages of an extraction were to concentrate endogenous

ligand from other materials, to improve specificity and to avoid damage to endogenous ligand or tracer with potentially damaging enzymes in the sample (Chard, 1978, cited in Suchinda Malaivijitnond, 1994). The double extraction could concentrate and recover much more amount of hormone from sample (Suchinda Malaivijitnond, 1994)

5.1 *Extraction of unknown plasma and quality control*

Two aliqouts of each sample (200 µl for female, and 500 µl for male) and 3 replicates of each quality control were transferred into cone tubes and reconstituted with 4 ml fresh diethyl ether. Four millilitres of fresh diethyl ether only was added to two tubes and labelled as ether blank. Every tubes was subjected to extraction under agitation machine for 3 minutes. The ether was allowed to settle. The tubes were dipped in 95 % ethanol containing chips of dry ice. An aqueous layer froze and the upper ether layer was decanted to an assay tube. Every tubes were repeated extraction with 4 ml diethyl ether. The second ether layer was poured over the first one in the assay tube and allowed to evaporate to dry in a heating block. The remaining residue was redissolved with 500 µl of assay buffer solution. The assay tubes were mixed under agitation machine for 1 minute, allowed to stand for 10 minutes then mixed again for 1 minute. This solution was then ready to be used for assay.

5.2 *Recovery of extraction*

Two aliquots of 500 µl and 200 µl of the female pooled plasma were added with 10 µl of recovery tracer in cone tubes, mixed and allowed to equilibrate with the tracer for 30-60 minutes at room temperature. The mixed aliquots were double extracted with fresh diethyl ether and separated for the ether layer as well as the unknown plasma. The ether was evaporated to dry in a heating block. Remaining residue was redissolved with 500 µl of assay buffer solution, and mixed like the unknown sample. The reconstitution was decanted to a counting vial. Two recovery totals were prepared by adding 10 µl of recovery tracer and 500 µl of assay buffer solution into assay tubes, then transferred to counting vials. Five millilitres of scintillation fluid was added to counting vials. After equilibration, each vial was counted in a β-counter for 5 minutes. Recovery of extraction was calculated by:

$$\text{Recovery of extraction (\%)} = \frac{\text{Sample recovery counts} \times 100}{\text{Recovery total counts}}$$

Recovery of extraction in this study ranged between 80.18 % and 80.95 %. This recovery factor was merely used for monitoring efficiency of extraction in each assay. It was not applied to calculation of estradiol concentration. Hence, an underestimation for estradiol level should be remarked.

Assay procedures:

Various reagents were added to yield a final volume of 900 µl in an assay tube. Three replicates of zero antigen or maximum binding (B0) tubes were prepared by adding 500 µl of assay buffer solution in assay tubes. One hundred microlitres of antiserum to estradiol was added to the B0, extracted unknown sample, quality controls, and serial dilutions of standard in 500 µl of assay buffer solution in assay tubes. Three non-specific binding (NSB) tubes were prepared by adding 600 µl of assay buffer solution in assay tubes. Three total count (TC) tubes were prepared by adding 800 µl of assay buffer solution in assay tubes. The latter two triplicates were prepared without added antiserum. A 100 µl amount of estradiol working tracer was added to each tubes, then mixed and incubated at 4°C for 18-24 hours. Contents of estradiol assay tubes are summarized in table 3-4.

Table 3-4 Summary of contents of assay tubes in estradiol assay.

	Buffer	**Standard or Sample**	**Antiserum to estradiol**	**Estradiol tracer**	*Incubate*	**Charcoal suspension**
Total count	800 µl	-	-	100 µl	*at*	-
NSB	600 µl	-	-	100 µl	*4 ℃*	200 µl
B0	500 µl	-	100 µl	100 µl	*18-24*	200 µl
Standard or Sample	-	500 µl	100 µl	100 µl	*hours*	200 µl

Except for the total count tubes, separation of antibody-bound hormone was performed in an ice bath by addition of 200 µl of cold charcoal suspension which was stirred continuously with magnetic stirrer. Each tube was mixed under agitation machine for 1 minute and allowed to stand for 30 minutes at 4°C, then centrifuged at 1,100 x g, 4°C for 15 minutes. The supernatant was decanted into a counting vial. Five millilitres of scintillation fluid was added to each vial, and allowed to equilibrate for 60 minutes. Each vial was counted in a β-counter for 5 minutes.

A dose response curve was plotted using $\log_{10}$ dose against standard bound-NSB (counts per minute), and regression analysis was performed. Result for each sample (fmol/tube) was read from the regression line of the standard curve. This result was transformed to concentration per millilitre (fmol/ml) according to volume of the sample. Then it was multiplied by molecular weight of estradiol (272.39) and 10^{-3} in order to express the concentration of estradiol as picogram per millilitre (pg/ml).

Validation of assay:

1. *Parallelism*

The high levels of softshell turtle estradiol were paralleled against the standard curve of human estradiol as shown in figure 3-5. There was no significant difference between the slopes of these two regression lines.

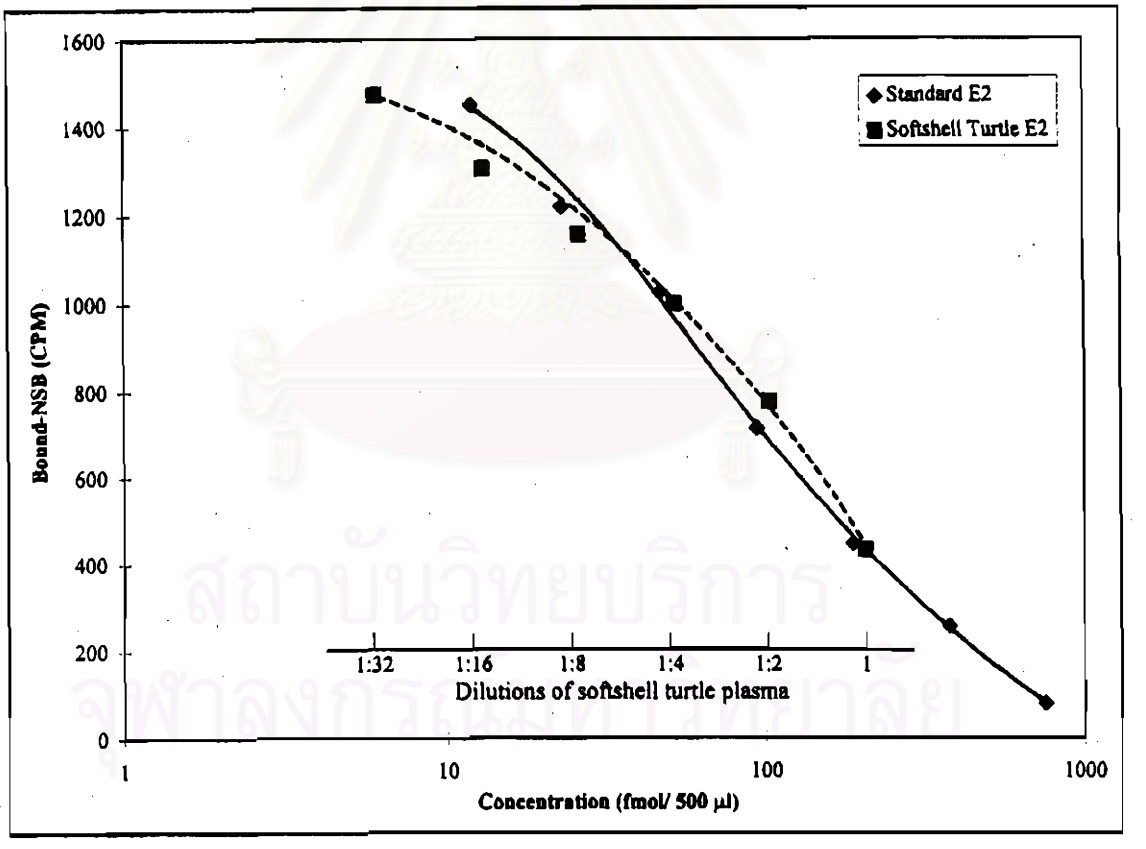

Figure 3-5 Parallelism of immunoreactivities of human estradiol standard and serial dilutions of softshell turtle plasma.

2. *Specificity*

The cross reactions of steroids likely to be present in plasma were tested by WHO matched reagent programme as listed below (Sufi, Donaldson, Jeffcoate, 1990).

- Estradiol %Cross reaction = 100.0
- Estriol %Cross reaction = 0.8
- Estrone %Cross reaction < 0.02
- Cortisol %Cross reaction < 0.02
- Progesterone %Cross reaction = 0.02
- Testosterone %Cross reaction < 0.02

3. *Sensitivity*

The sensitivity of estradiol assay was 1.57 pg/ml.

4. *Precision*

The intra-assay and inter-assay variation of estradiol assay are presented as coefficients of variation in table 3-5.

Table 3-5 Precision of estradiol assay.

Quality Control	Mean (fmol / 200 µl)	Coefficient of Variation	
		Intra-assay (n=10)	Inter-assay (n=30)
Males	22.41 ± 3.33	4.45 - 9.67	14.86
Females	124.05 ± 15.44	7.36 - 9.09	12.45

5. *Accuracy*

The Pearson's correlation coefficient between the determined and added values of estradiol was 0.969 ($p < 0.01$).

3.3.3 Progesterone assay

To perform radioimmunoassay for progesterone of softshell turtles, progesterone of the softshell turtles was allowed to compete with (1, 2, 6, 7-^{3}H) progesterone in binding to mouse antibody to progesterone-3CMO-BSA. Dextran-charcoal was utilized to separate free hormone from antibody-bound hormone. The following method procedures followed the WHO matched reagent programme (Sufi, Donaldson and Jeffcoate, 1990).

Reagent preparation:

1. *Progesterone antiserum*

Progesterone antiserum was produced in mice by monoclonal antibody technique in response to progesterone-3CMO-BSA conjugate. It was provided in lyophilized form. The freeze dried antiserum is stable for several years if stored at 4°C. The content of one bottle of antiserum was reconstituted with 10 ml assay buffer solution, allowed to stand for 5-10 minutes and mixed before use with a final dilution of 1: 210,000 in assay tubes.

2. *Progesterone tracer*

Solution of (1, 2, 6, 7-^{3}H) progesterone that was provided in the concentration of 3.7 MBq (100 μCi) was transferred from sealed ampule to a 10 ml volumetric flask. The ampule was rinsed with a solution of toluene: ethanol (9:1 by volume), and the washings was added to the contents of the flask. The volume of the stock solution was made up to 10 ml with the toluene: ethanol (9:1) solution. The stock solution with concentration of 370 kBq or 10 μCi/ml was stored in a dark bottle at -20°C.

Working tracer was prepared as follows: 400 μl of the stock solution was transferred into a bottle and the solvent was allowed to evaporate. The residue was redissolved with 12 ml assay buffer solution. The bottle was allowed to stand for 30 minutes in order for resolvation of the tracer.

3. *Progesterone standard*

This was provided in ethanolic solution at a concentration of 250 nmol/l. An ampule and pipettes to be use were cooled to 4 °C. After the ampule was opened, aliquots of 3 or 4 x 100 μl were transferred to the 15 ml bottles. These aliquots were stored at 4°C until needed. When required, 10 ml assay buffer solution was added to the bottleand heated to 40°C in a water bath for 30 minutes. The solution was mixed vigorously and allowed to cool to 4°C before use. This solution (solution B) contained 2.5 nmol/l or 2,500 fmol/ml progesterone and was stable for 2-3 weeks when stored at 4°C. Upon assay, the solution B was serially diluted to obtain the following triplicate standard dose levels: 1250, 625, 313, 156, 78, 39 and 20 fmol/500 μl.

4. *Recovery tracer*

Recovery tracer was prepared to monitor the recovery of extraction. Four hundred microlitres of stock tracer was transferred to a vial, the solvent was allowed to evaporate and redissolved in 1.2 ml assay buffer solution.

5. *Sample preparation*

Plasma of softshell turtle was extracted with diethyl ether prior to assay. The advantages of an extraction were to concentrate endogenous ligand from other materials, to improve specificity and to avoid damage to endogenous ligand or tracer with potentially damaging enzymes in the sample (Chard, 1978, cited in Suchinda Malaivijitnond, 1994).

5.1 *Extraction of unknown plasma and quality control*

Two aliqouts of each sample (50 µl for female and 500 µl for male) and 3 replicates of each quality control were transferred into cone tubes and reconstituted with 5 ml fresh diethyl ether. Five millilitres of fresh diethyl ether only was added to two tubes and labelled as ether blank. Every tubes was subjected to extraction under agitation machine for 3 minutes. The ether was allowed to settle. The tubes were dipped in 95 % ethanol containing chips of dry ice. An aqueous layer froze and the upper ether layer was decanted to an assay tube. The ether was evaporated to dry in a heating block. The remaining residue was redissolved with 500 µl of assay buffer solution. The assay tubes were mixed under agitation machine for 1 minute, allowed to stand for 10 minutes then mixed again for 1 minute. This solution was then ready to be used for assay.

5.2 *Recovery of extraction*

Two aliquots of 50 µl of the female pooled plasma were added with 10 µl of recovery tracer in cone tubes, mixed and allowed to equilibrate with the tracer for 30-60 minutes at room temperature. The mixed aliquots were extracted with fresh diethyl ether and separated for the ether layer as well as the unknown plasma. The ether was evaporated to dry in a heating block. Remaining residue was redissolved with 500 µl of assay buffer solution, and mixed like the unknown sample. The reconstitution was decanted to a counting vial. Two recovery totals were prepared by adding 10 µl of recovery tracer and 500 µl of assay buffer solution into assay tubes, then transferred to counting vials. Five millilitres of scintillation fluid was added to counting vials. After equilibration, each vial was counted in a β-counter for 5 minutes. Recovery of extraction was calculated by:

$$\text{Recovery of extraction (\%)} = \frac{\text{Sample recovery counts}}{\text{Recovery total counts}} \times 100$$

Recovery of extraction in this study ranged from 60.35 to 60.57 %. This recovery factor was merely used for monitoring efficiency of extraction in each assay. It was not applied to calculation of progesterone concentration.

According to Sufi, Donaldson and Jeffcoate (1990), it was essential to make sure that the extract is properly dissolved before analysis. In case of progesterone it might be necessary to heat the reconstituted extract at 50°C for 15-40 minutes to ensure that the progesterone was completely dissolved. But after many trials, it was found that heating assay tube which was not single use could dissolve uncontrol impurities resulted in unacceptable poor precision. Progesterone assay was thus conducted without heating the assay tube. Low recovery of extraction was compensated by better precision. By the way, an underestimation for progesterone assay should be remarked.

Assay procedures:

Various reagents were added to yield a final volume of 900 µl in an assay tube. Three replicates of zero antigen or maximum binding (B0) tubes were prepared by adding 500 µl of assay buffer solution in assay tubes. A 100 µl amount of antiserum to progesterone was added to the B0, extracted unknown sample, quality controls, and serial dilutions of standard in 500 µl of assay buffer solution in assay tubes. Three non-specific binding (NSB) tubes and three total count (TC) tubes were prepared by respectively adding 600 µl and 800 µl of assay buffer solution in assay tubes. The latter two triplicates were prepared without added antiserum. A 100 µl amount of progesterone working tracer was added to each tube, then mixed and incubated at 4°C for 18-24 hours. Contents of progesterone assay tubes are summarized in table 3-6.

Table 3-6 Summary of contents of assay tubes in progesterone assay.

	Buffer	Standard or Sample	Antiserum to progesterone	Progesterone tracer	*Incubate*	Charcoal suspension
Total count	800 µl	-	-	100 µl	*at*	-
NSB	600 µl	-	-	100 µl	*4 ℃*	200 µl
B0	500 µl	-	100 µl	100 µl	*18-24*	200 µl
Standard or Sample	-	500 µl	100 µl	100 µl	*hours*	200 µl

Separation of antibody-bound hormone was performed in an ice bath by addition of 200 µl of cold charcoal suspension which was stirred continuously with magnetic stirrer except for the total count tubes. Each tube was mixed under agitation machine for 1 minute and allowed to stand for 30 minutes at 4°C, then centrifuged at 1,100 x g, 4°C for 15 minutes. The supernatant was decanted into a counting vial. Five millilitres of scintillation fluid was added to each vial, and allowed to equilibrate for 60 minutes. Each vial was counted in a β-counter for 5 minutes.

A dose response curve was plotted using $\log_{10}$ dose against standard bound-NSB (counts per minute), and regression analysis was performed. Result for each sample (fmol/tube) was read from the regression line of the standard curve. This result was transformed to concentration per millilitre (fmol/ml) according to volume of the sample. It was then multiplied by molecular weight of progesterone (314.47) and 10^{-6} in order to express the concentration of progesterone as nanogram per millilitre (ng/ml).

Validation of assay:

1. *Parallelism*

Due to limited amount of softshell turtle plasma, only medium level of progesterone was available. Dilution curve of softshell turtle plasma containing medium level of progesterone was paralleled against the standard curve of human progesterone as shown in figure 3-6. There was no significant difference between the slopes of these two regression lines.

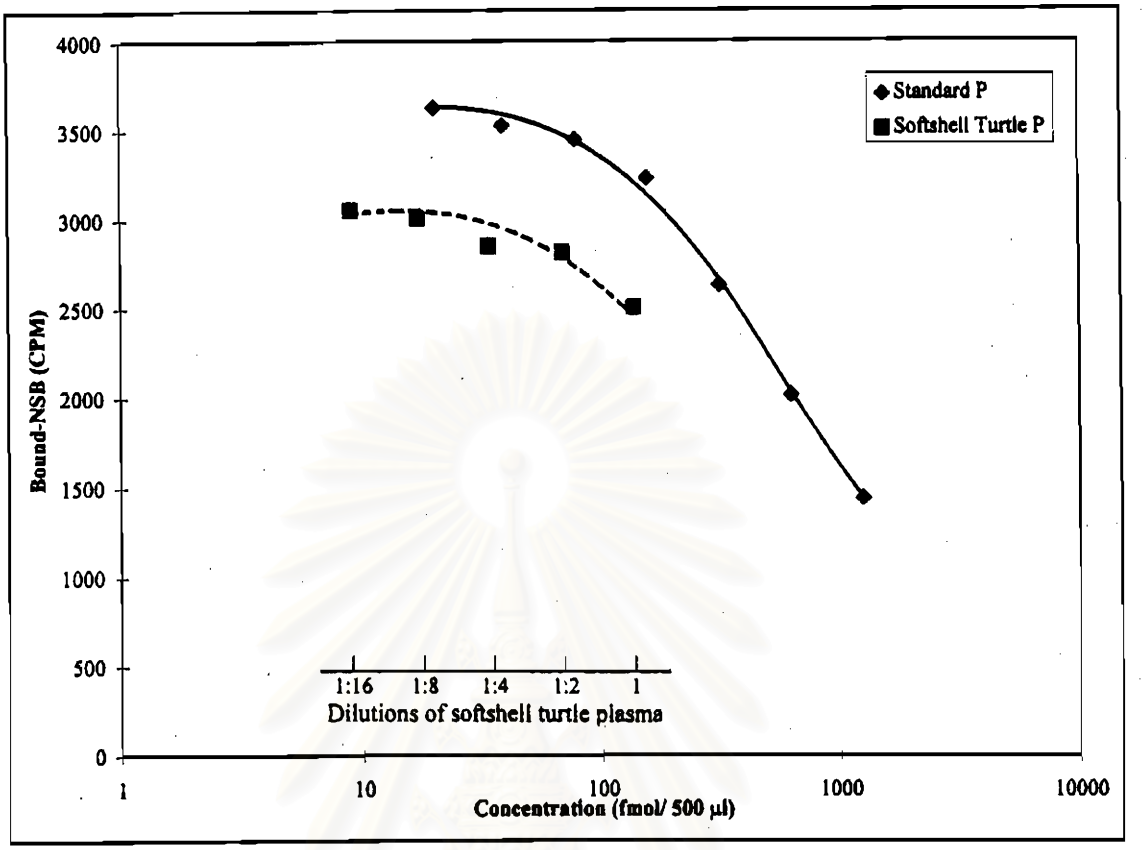

Figure 3-6 Parallelism of immunoreactivities of human progesterone standard and serial dilutions of softshell turtle plasma.

2. *Specificity*

The cross reactions of steroids likely to be present in plasma were tested by WHO matched reagent programme as follows (Sufi, Donaldson, Jeffcoate, 1990).

- Progesterone %Cross reaction = 100.0
- Cortisol %Cross reaction = 0.002
- Testosterone %Cross reaction = 0.02
- 17α Hydroxyprogesterone %Cross reaction = 1.6
- 20α Dihydroprogesterone %Cross reaction = 0.04

3. *Sensitivity*

The sensitivity of progesterone assay was 7.52 pg/ml.

4. *Precision*

The intra-assay and inter-assay variation of progesterone assay are presented as coefficients of variation in table 3-7.

Table 3-7 Precision of progesterone assay.

Quality Control	Mean (fmol / 50 µl)	Coefficient of Variation	
		Intra-assay (n=10)	Inter-assay (n=30)
Males	348.22 ± 50.53	8.70 - 9.75	14.51
Females	373.96 ± 51.37	8.08 - 9.46	13.74

5. *Accuracy*

The Pearson's correlation coefficient between the determined and added values of progesterone was 0.921 ($p < 0.01$).

3.3.4 Analysis

Annual reproductive cycles of male and female softshell turtle were analyzed according to the following two categories.

1. Temporal changes in hormonal levels

Levels of steroids of each sex in each sampling, monthly levels as well as seasonal levels were calculated for mean and standard error of the mean (S.E.M.). The means were compared among samplings, months and season using analysis of variance (ANOVA), and Duncan's multiple range test was used to classify homogeneous subsets of the means. Probability of $p \leq 0.05$ was considered to be significantly different.

2. Correlation to climatic factors

Mean levels of each samplings, months and seasons were analyzed for correlation with climatic data of Bangkok area (Meteorological department, 1997) by bivariate correlation method. Significant correlation was considered from probability of $p \leq 0.05$.

General calculations were performed on computer by Microsoft Excel for Windows 95 version 7.0. Statistical analysis were performed on computer by SPSS for Windows release 7.0.