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CHAPTER VII

APPENDICES

APPENDIX I : CHEMICAL REAGENTS

Radioimmunoassay Kits

- Total T_4 double antibody kit (Cat No. KT4D1, KT4D5)	:	Diagnostic Products Corporation,Los angeles, USA.
- TSH double antibody kit (Cat No. CA 1591)	:	INSTAR Corporation, Stillwater, USA.
- PRL double antibody kit (Cat No. KPRD1, KPRD5)	:	Diagnostic Products Corporation,Los angeles, USA.
- estradiol-17B standard	:	WHO RIA Reagent Programme.
- estradiol-17B antiserum	:	WHO RIA Reagent Programme.
- (2,4,6,7-^{3}H) estradiol	:	Amersham International PLC, England.
- cortisol standard	:	WHO RIA Reagent Programme.
- cortisol antiserum	:	WHO RIA Reagent Programme.
- (1,2,6,7-^{3}H) cortisol	:	Amersham International PLC, England.
- testosterone standard	:	WHO RIA Reagent Programme.
- testosterone antiserum	:	WHO RIA Reagent Programme.
- (1,2,6,7-^{3}H) testosterone	:	Amersham International PLC, England.

Hormones

- L-thyroxin (Lot. 70H0673) : Sigma Chemical Co., St.Louis, USA.
- 3,3',5-triiodo-L-thyronine (Cat No. T5516) : Sigma Chemical Co., St.Louis, USA.
- thyrotropin releasing hormone (Cat No. P-2161) : Sigma Chemical Co., St.Louis, USA.

Others

- charcoal : WHO RIA Reagent Programme.
- dextran : WHO RIA Reagent Programme.
- gelatin : Difco Laboratories, Michigan, USA.
- sodium dihydrogen phosphate monohydrate : Merck Ltd, Darmstadt, Germany.
- disodium hydrogen phosphate : Merck Ltd, Darmstadt, Germany.
- sodium chloride : Merck Ltd, Darmstadt, Germany.
- phenyl-oxazolylphenyl-oxazolyl-phenyl : Sigma Chemical Co., St.Louis, USA.
- 2,5-diphenyloxazole(PPO) : Sigma Chemical Co., St.Louis, USA.
- toluene : Merck Ltd, Darmstadt, Germany.
- 1,4-dioxane : Merck Ltd, Darmstadt, Germany.
- thimerosol : Sigma Chemical Co., St.Louis, USA.
- methanol : Merck Ltd, Darmstadt, Germany.
- ethanol absolute : Merck Ltd, Darmstadt, Germany.
- diethyl ether : Merck Ltd, Darmstadt, Germany.
- normal saline(0.9% NaCl) : Abbott Pharma Ltd., Bangkok, Thailand.

- celite	:	Fluka Chemei AG, Switzerland.
- sodium hydroxide	:	BDH Chemical Ltd, England.
- heparin	:	Leo Pharmaceutical Products, Balelrp, Denmark.
- ketamine hydrochloride	:	Parke Davis PTV, Ltd, Sydney, Australia.
- [N-methyl-^{3}H] morphine	:	Du Pont Company, Wilmington, USA.

APPENDIX II : INSTRUMENTS

- needle and syringes	:	Terumo Corporation, Tokyo, Japan.
- mixer	:	Thermolyne Corporation, Dubugelowa, USA.
- pH-meter	:	Corning-EEI Scientific Instruments, England.
- magnetic stirrer	:	S-18521 Thermolyne Corporation, Iowa, USA.
- balance	:	W.M. Ainsworth and Sons Inc., Benver, USA.
- refigerated centifugator	:	International Equipment Company Mass, USA.
- autoclave	:	Forma Scientific Mariette, Ohio, USA.
- ultrasonic cleaner	:	W.M. Ainsworth and Sons Inc., Benver, Colo, USA.
	:	Elma D-7700, West Germany.
- dri-block heater	:	Thecam Incorporated, Princeton, N.J., USA.

- adjustable micopipette : Nichiryo Co., Ltd., Tokyo, Japan.
  model 5000
- micropipette : Eppendrof 3130, Germany.
- repetitive syringe dispenser : Nichiryo Co., Ltd., Tokyo, Japan.
  model 8100
- water bath : Lab-line Instruments, Inc.,
  Melrose Park, Illinois, USA.
- B-liquid scintillation counter : Packard Instrument Co., USA.
- gamma-counter model 1280 : LKB Wallac, Finland.
- millipore size 0.22 um : Corp Bedford Mass, USA.

APPENDIX III : REAGENT PREPARATIONS

Morphine Solution

Morphine stock solution was freshly prepared once a week. The weighted morphine hydrochloride powder for one week treatment (M.W. : 321.8 : kindly supplied by Drug Depedence Research Center, Institute of Health Research, Chulalongkorn University) was completely dissolved in tri-distilled water to a final volume of 0.5 ml/day. The solution was steriled by filtering through 0.22 um millipore filter and stored in a dark bottle at room temperature.

Radioactive morphine solution

One hundreds microliters of radioactive morphine (N-methyl-^{3}H-morphine : MW. 285.3 : DuPont Company, USA) was aliquoted from stock solution (1 mCi/ml or 0.0038 mg/ml) that was provided in

ethanol solution into a dark vial and dried under the sterile air flow. The practice of isotope dilution involved adding a small amount of radioactive drug (approximately 1 percent of the total) to a system already containing the unlabelled drug (Curry and Whelpton, 1983). Then, the remaining residual was redissolved with 2.5 ml sterile saline and added 0.01 mg morphine hydrochloride powder. This solution was homogenously mixed in vortex mixer. The final concentration of radioactive morphine was obtained as 20 uCi/ 0.5 ml.

Preparation of Steroid Assay Reagents

These reagent preparation followed WHO(1990) procedure.

1. Steroid assay buffer (buffer S)

*2.35 g sodium dihydrogen phosphate(anhydrous) : NaH_2PO_4

*11.6 g disodium hydrogen phosphate(anhydrous) : Na_2HPO_4

8.8 g sodium chloride : NaCl

0.1 g thiomersol(merthiolate)

1.0 g gelatin

*If the hydrated form are used then the amounts taken must be increased in proportion to the degree of hydration.

Dissolved all constituents except gelatin in 750 ml distilled water. The gelatin must be dissolved in a small volume of warm, not boil, water before being added to the other reagents. Made up the volume to 1 liter. The pH of this buffer should be check to be between 7.2 and 7.4 by drop wise addition of sodium hydroxide (NaOH) or hydrochloric acid (HCl).

The buffer could be stable at 4 ^{o}C for at least 1 month. This buffer S was used as the diluent for all reagents in steroid assays.

2. Charcoal suspension

0.625 g charcoal

0.0625 g dextran

100 ml buffer S

Dissolved dextran in 100 ml buffer S in a stoppered container, then added charcoal and shaked vigorously for 30 seconds. The charcoal reagent should be stable at 4 ^{o}C for up to one month. The settle down suspension should be stirred vigorously during use.

3. Scintillation fluid

5.0 g 2,3-diphenyloxazole (PPO)

0.3 g 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP)

1.0 l toluene

200 ml dioxane

Homogenously mixed all constituents and stored in the dark bottle. The solution could be stable at the room temperature.

APPENDIX IV : QUALITY CONTROL PREPARATION

Hormone Free Serum(HFS)

Hormone free serum was used as diluent adjusting the quality control pool serum and prepared following the North East Thames Region Immunoassay Unit (NETRIA). It was performed by filtering

pooled monkey serum through the column. The column was made from the 50 ml dispossable syringe shown in Figure 58. The bottom is paked with glass wool, and consequently, covered with filtered paper (Whatman no.4), celite and, charcoal and celite solution (4:1). The celite and charcoal solution was made by mixing 8 g charcoal and 2 g celite with 20 ml distilled water and then pouring into the column and eluted with 5 ml distilled water. After a column was settled, 50 ml of pooled monkey serum adding 2,000 cpm/ml ^{125}I-T_4 was then filtered through the column. The hormone free serum was then collected after the estimated water volume was already elute. The eluated serum was tested with ammonium salt ($(NH_4)_2SO_4$). The ammonium salt could have a reaction with protein in serum and represented a white precipitation. From this method it is possible to separate over 98% of endogenous hormones which monitored by counting ^{125}I-T_4.

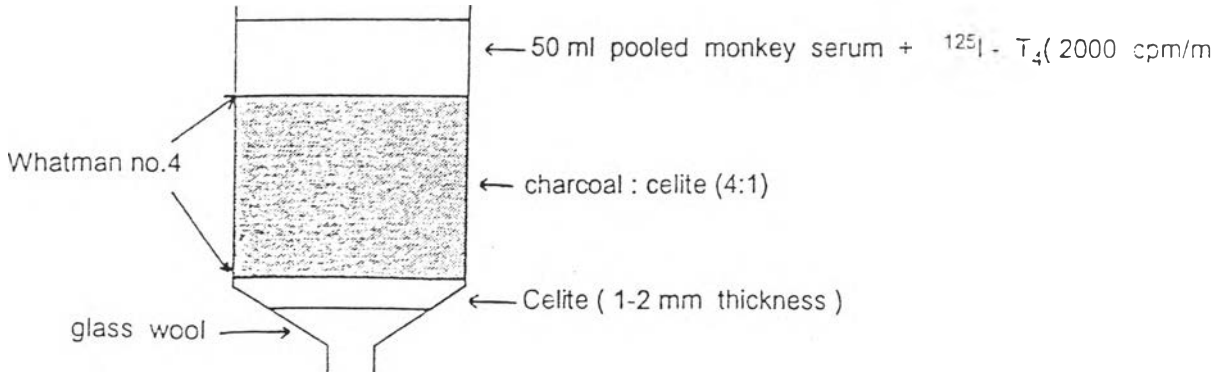

Figure 56 A diagram showing column preparation for performing HFS.

High Hormonal Level of Quality Control (QC)

1. High PRL level of quality control

Hyperprolactinemia in cynomolgus monkeys were stimulated by intravenous injection of synthetic TRH (M.W. 362.4 : Sigma Chemical Co., USA). The used dose was based on the stimulation of PRL release by TRH in man (Jacobs et al., 1971; Burrow, Spaulding and Donabedian, 1977; Namking, et al., 1982), rhesus monkeys (Diefenbach, et al., 1976) and rats (Buydens, et al., 1987). Moreover, the baseline PRL level was higher in females than in males and the peak level response to TRH administration in females was also higher than in males (Namking, et al., 1982). Therefore, adult female cynomolgus monkeys were considered to be a subject for preparing high PRL level of quality control.

At first, two adult female monkeys were administered a 100 ug bolus intravenous injection of TRH in 1.0 ml saline, blood sample were collected frequently at 0, 10, 20, 30, 45, 60, 90, 120, and 180 minutes, thereafter. Serum PRL levels were measured by homogenous radioimmunoassay system (human PRL RIA kit : Diagnostic Products Corporation, USA). A striking and consistent elevation of PRL levels was seen 10 minutes after intravenous TRH injection. The peak values occurred at 10 to 30 minutes and thereafter serum PRL levels fell slowly toward baseline by 180 minutes (Figure 57). Furthermore, from this result, ten adult female monkeys were given 100 ug TRH as a bolus intravenous injection in 1.0 ml saline and blood withdrawal was followed at 10 minutes after injection. Separated blood serum of each monkey were pooled together and

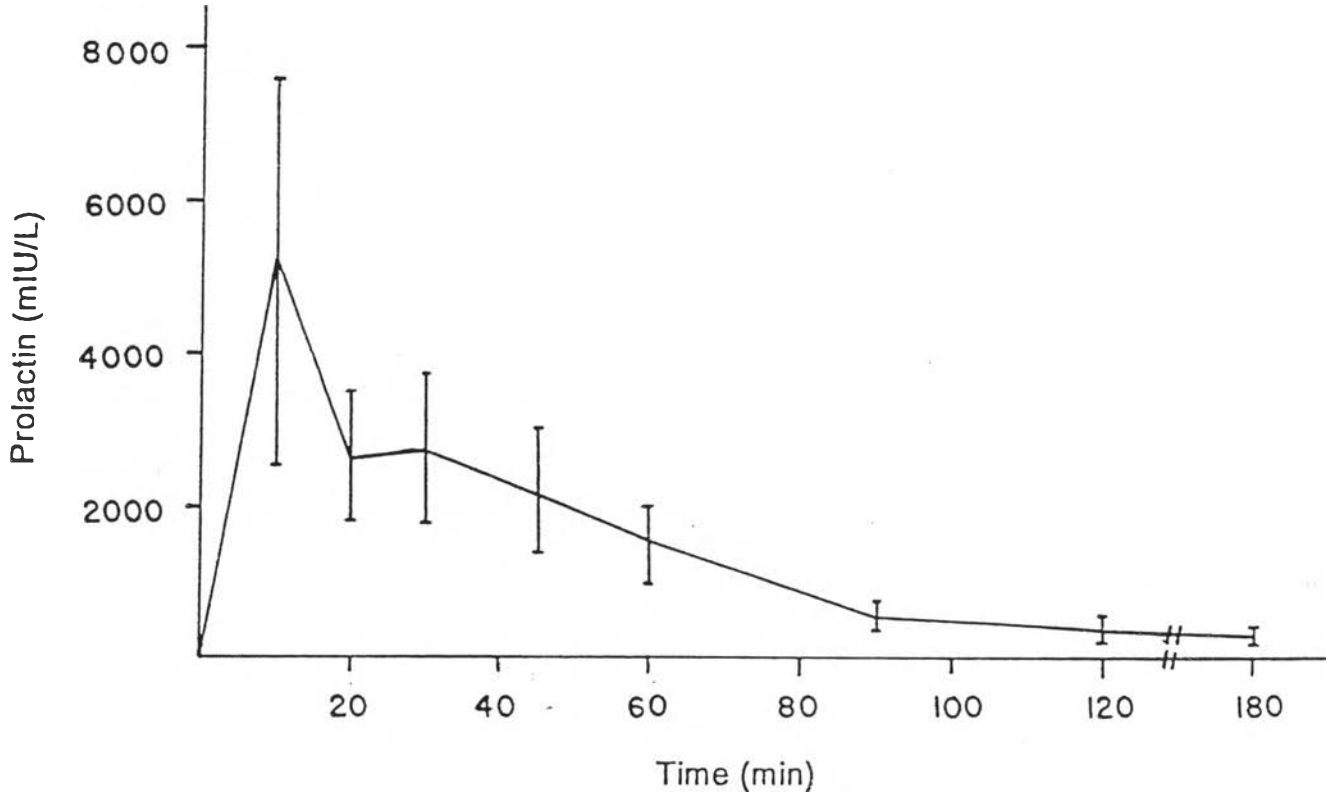

Figure 57 Mean prolactin profile(X±SE) in adult female monkeys after an intravenous administration of 100 ug TRH.

determined PRL level thereafter. The high PRL level of quality control was adjusted from pooled PRL serum to be 1677.15 ± 46.96 mIU/l concentration or at 20 percent bound of standard curve by using serial dilution with pooled normal serum of known PRL concentration.

2. High T_4 level of quality control

Pool monkey serum of high T_4 level was prepared by adding standard thyroxin (Sodium L-thyroxin pentahydrate : Sigma Chemical Co., USA) to pool normal monkey serum. From stock solution of standard T_4 (1 mg/ml), it was diluted with tri-distilled water (1 : 10) to obtain the working solution in concentration of 100 ug/ml. In small amount, this working solution was diluted in a serial set with pool normal monkey serum which occupied T_4 concentration ranging between 4.5 ug/dl and 6.0 ug/dl. The optimal ratio of serial dilution was then selected (1 : 16) and used for preparing the pool serum of high T_4 level (about 16 ug/dl or 20 percent intercept of standard curve) in the large scale. This prepared pool serum was aliquoted into each tube to avoid repeated freezing and thawing.

Low Hormonal Level of Quality Control

The serial dilution of pool normal monkey serum and hormone free-serum was done in the small scale. The optimal ratio of serial dilution that represented the hormonal concentration about 80 percent intercept of standard curve was then selected to prepare the quality control serum in the large scale. At the onset of assay, TSH, PRL and T_4 concentrations in the low values were provided in 2.79 ±

0.11 mIU/L, 113.71 ± 8.99 mIU/L and 1.72 ± 0.07 ug/dl, respectively.

APPENDIX V : TERMINOLOGY

1. **Agonists** are introduced to many drugs mimic at least some of the effects of such endogenous compounds by interaction with the appropiate physiological receptor.

2. **Antagonists** are desinated as compounds that are themselves devoid of intrinsic pharmacological activity but cause effects by inhibition of the action of a specific agonist e.g. by competition for agonist binding sites.

3.**Opiate** is once used to desinate drug derived from opium such as morphine, codeine, and the many semisynthetic congeners of morphine.

4. **Opioid** is refered in a generic sense to all drugs, natural and synthetic, with morphine like actions. In such contex opiate and opioid are interchangable. More recently, opioid has also been used to refer to antagonists of morphine-like drugs as well as to receptors or binding sites that combine with such agents.

5. Physical dependence refers to an altered physiological state (neuroadaptation) produced by the repeated administration of a drug, which necessitates the continued administraton of the drug to prevent the appearance of a stereotrypical syndrome, the withdrawal or abstinence syndrome.

6. **Tolerance** has developed when, after repeated administration, a given dose of a drug produces a decreased effect or, conversely, when increasingly larger doses must be administered to obtain the effects observed within the original dose. It is possible to distinguish two

varieties of acquired pharmacological tolerance : dispositional and pharmacodynamic.

7. **Dispositional tolerance** results from change in the pharmacokinetic properties of the agent in the organism, such that reduced concentrations are present at the sites of drug action. The most common mechanism is an increase rate of metabolism. Dispositional tolerance has relatively little effect on the peak intensity of action and does not usually result in more than a three fold decrease in sensitivity.

8. **Pharmacodynamic tolerance** results from adaptive changes within affected systems, such that response is reduced in the presence of the same concentration of the drug.

9. Rebound or overshoot phenomena can postulate by enzyme induction theories that drugs that cause dependence could directly or indirectly inhibit an enzyme that synthesized a product important for cell activity (e.g., a neurotransmitter), and that the level of the enzyme itself is regulated by its product, the neurotransmitter. The initial drug effect is a result of the decrease in transmitter concentration, but this decrease also leads to increase synthesis of the enzyme and a new steady-state level that restores transmitter concentration, resulting in tolerance. When the drug is withdrawn there is excess enzyme, which then causes excess synthesis of transmiter, and this produces rebound effects until the enzyme activity falls to a new steady state (Gilman et al., 1985).

10. **Homologous radioimmunoassay** an assay will be called "homologous" when the hormone used for immunisation and for labelling are of the same animal species.

11. **Heterologous radioimmunoassay** an assay will be called "heterologous" where one is using an antiserum of one specie in combination with a labelled hormone of a second specie.

12. **Homogenous radioimmunoassay** an assay will be called "homogenous" since the material used for labelling and for immunisation was of the same species but either different species or different hormones from unknown sample (L'Hermite, 1973).

BIOGRAPHY

Miss Suchinda Malaivijitnond was born on August 10, 1967 at Saraburi province. She recieved the Bachelor Degree of Science (Biology, Second Class Hornor) from Khon Kaen University in 1988, and the Master Degree of Science (Zoology) from Chulalongkorn University in 1990.