II Materials and Methods

II.1 Chemicals

The chemicals below are listed in groups according to the com - pany's name where they were brought from :

II.1.1 Sigma Chemical Co, USA

Acrylamide, bovine serum albumin fraction V, citric acid, Coomassie brilliant blue R, sodium dodecylsulfate (SDS), 2 - mercaptoethanol , N , N′ - methylene - bis - acrylamide, O - phenylenediamine (OPD), sodium borohydride , sodium citrate and Tween 20.

II.1.2 Merck, West Germany

Copper sulfate , disodium hydrogenphosphate , ethylenediamine tetraacetic acid disodium (EDTA), glycine, hydrochloric acid , hydrogen peroxide, sodium acetate, sodium chloride, sodium dihydrogenphosphate, sulfuric acid and Tris (hydroxymethyl) - aminomethane.

II.1.3 May & Baker Ltd , England

Ammonium persulfate, Bromophenol blue, potassium dihydrogenphosphate, sodium bicarbonate and sodium barbital.

II.1.4 Pharmacia Fine Chemical , Sweden

Blue dextran 2000 , Sephadex G.150 , and Sepharose 4B.

II.1.5 Fluka , Switzerland

Patassium sodium tartrate , sodium hydroxide , sodium periodate and N,N,N′,N′ - tetramethyleneethylenediamine (TEMED).

II.1.6 Others

Ammonium sulfate (Chiatai , Thailand)

Biogel P-60 (BIO - RAD Laboratories, West Germany)

Ethanol 95 % (Ayuttaya Alcohol Factory, Thailand)

II. 2 Materials

- β - HCG EIA kits (Hoffman La Roche, Switzerland)
- β - HCG latex agglutination kits (Rama Hospital , Bangkok)
- Horseradish peroxidase , type II (Sigma Chemical Co , USA)
- Polystyrene tubes , 12 x 75 cm (Costar , Cambridge , England)
- Serum samples for HCG determination were collected from trophoblastic patients admitted in Maharaj Nakorn Chiang Mai Hospital , Faculty of Medicine , Chiang Mai University and kept at -20 °C until use.
- Normal serum samples were obtained from 20 each of healthy men and women , aged between 21 and 64 years and stored at -20 °C until use.
- Standard HCG (Profasi Serono , Italy)
- Rabbit anti - β - HCG (DAKO, USA)

II.3 Experimental Instruments

- High speed centrifuge (Sorvall , USA)
- Disc - gel electrophoretic apparatus No.51515 (Gelman Instrument Co., USA)
- Incubator (Heraeus , West Germany)

- Power supply (Gelman Instrument Co , USA)
- Fraction collector , Frac 100 (Pharmacia Fine Chemical , Sweden)
- UV - visible spectrophotometer , Ultrospec 4050 (LKB Biochrom, England)
- Vacuum pump (General Electric , USA)

II.4 General Methods.

II.4.1 Analysis of Protein Concentration by Lowry's Method. (60)

Reagents

1. Solution A : 2 % (w/v) sodium - potassium tartrate
2. Solution B_1 : 1 % (w/v) copper sulfate
3. Solution B_2 : 2 % (w/v) sodium carbonate in 0.1 m NaOH
4. Working solution , was freshly prepared by mixing solutions , A , B_1 and B_2 at ratio 10:1:1
5. Folin - phenol solution : 0.1 N
6. Standard protein solution : 1 mg/ml of bovine serum albumin

Procedure

Two hundred µl of various concentrations of bovine serum albumin in distilled water (0, 50, 100, 200, 400 and 600 µg/ml) or sample was placed into each tube. Two ml of working solution was added into all tubes , then the tubes were mixed gently and kept at room temperature for 10 min. After then , 200 µl of 0.1 N Folin - phenol solution was added into all tubes , mixed gently and kept at room temperature for 30 min. The O.D. at 500 nm of standards and sample were measured with a zero reagent blank. A standard protein curve is presented in Figure II.1 from which the protein content in the sample was determined.

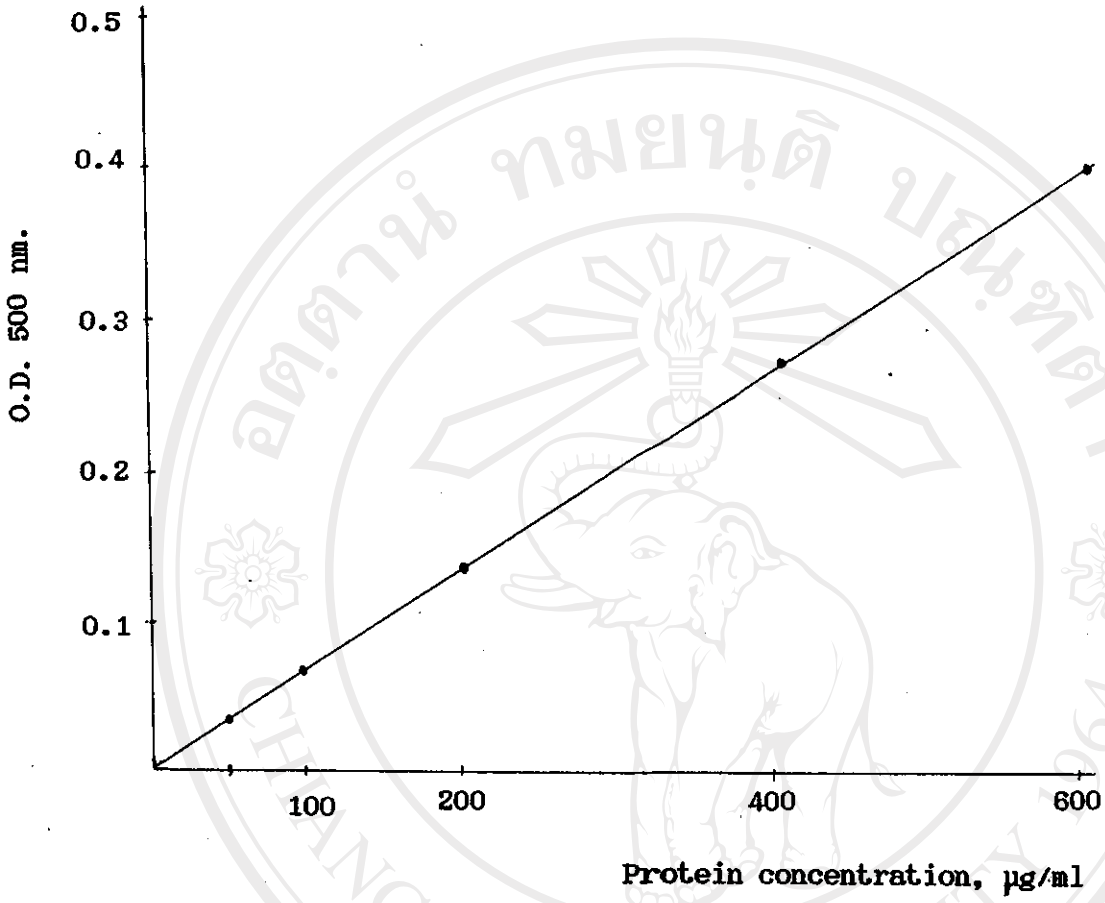


Figure II.1 Standard Protein Curve by Lowry's Method.

II.4.2 Analysis of Protein Purity by Polyacrylamide Gel Electrophoresis. (61)

Reagents

1. Stock bis - acrylamide : (0.8 : 30.0) % (w/v)
2. Separating gel buffer : 0.3 M Tris - HCl, pH 8.8
3. Stacking gel buffer : 0.5 M Tris - HCl, pH 6.8
4. Sample buffer : 0.001 M EDTA , 0.01 M Tris , 20 % glycerol and 0.2 % Bromophenol blue
   In the case of SDS - PAGE , SDS and 2 -mercaptoethanol were added to 1 % and 5 % , respectively.
5. Electrophoresis buffer : 0.025 M Tris and 0.192 M glycine,pH 8.3. In the case of SDS - PAGE , SDS was added to 0.1 %.
6. N,N,N′,N′ - Tetramethyleneethylenediamine (TEMED) : 0.5 %(v/v)
7. EDTA : 0.2 M
8. Ammonium persulphate : 10 % (w/v)
9. Staining solution : 0.25 % Coomassie - brilliant blue R , 10 % acetic acid and 50 % methanol
10. Destaining solution : 7 % acetic acid and 5 % methanal

Procedure

The 7.5 % total acrylamide (% T) and 2.5 % crosslink (% C) disc - polyacrylamide gel electrophoresis (PAGE) firstly described by Laemmli (61) , was prepared by mixing 1.5 ml of stock bis - acrylamide , 0.75 ml of separating gel buffer, 0.6 ml of 0.5 % TEMED, 0.06 ml of 0.2

M EDTA, 3.06 ml of distilled water and 0.03 ml of 10 % ammonium persulfate. After mixing 2.2 ml of the solution was loaded to each glass tube column (0.6 x 10 cm) standing on vertical of fixed stand and the bottom end was wrapped with many layers of Parafilm to prevent leakage of acrylamide solution, and then overlaid with distilled water. Polymerization was completed in 30 min and checked by observing the formation of sharp minicus between the gel and water , and then water on the top of polymerized gel was discarded. The stacking gel (3.0 % T) was also prepared by mixing 0.5 ml of stock bis - acrylamide , 1.0 ml of stacking gel buffer , 0.4 ml of 0.5 % TEMED , 0.02 ml of 0.2 M EDTA , 2.04 ml of distilled water and 0.04 ml of 10 % ammonium persulfate. After mixing , 0.4 ml of stacking gel solution was overlaid on the top of the polymerized gel , and then followed with distilled water.

After completion of polymerization , the gel tubes were inserted into the electrophoretic unit, and then electrophoresis buffer was laid into both the bottom (anode) and upper (cathode) chambers. After then , a constant current of 3 mA per gel tube was applied to pre-equilibrate for 15 min. Twenty - five µl of protein sample and 25 µl of sample buffer were mixed gently, and then applied on the top of each electrophoretically pre - equilibrated gel tube. After sample application , 3 mA per gel tube was applied until the Bromophenol blue marker pass the lenght of stacking gel , and then 5 mA per gel tube was used. After dye marker had been run to within 0.5 cm from the bottom of gel , then the

gel tubes were taken out from electrophoretic unit. The gel rods were pulled out from the tube supporters by injection of tapped water.

The gel rods were placed in staining solution for 2 - 3 hr at room temperature , then the gels were transferred into destaining solution. Destaining was done at room temperature with frequent changes of destaining solution until protein bands were clear from the background. The gels were kept in 7 % acetic acid. (v/v)

In case of SDS - PAGE , the same procedure was carried out but 0.1 % SDS was added to stacking and separating gel preparations. Sample buffer containing 1 % SDS and 5 % 2 - mercaptoethanol was mixed with protein sample and boiled at 100 °C for 5 min. The electrophoresis was run using buffer containing 0.1 % SDS.

II.4.3 Detection of HCG Activity by Agglutination Assay. (62)

Reagents

1. Latex coated with rabbit anti - β - HCG : in phosphate saline buffer , pH 7.0
2. Standard HCG in phosphate saline buffer , pH 7.0 : 1 i.u/ml (2^{nd} I.S. for the immunoassay)

Procedure

Fifty µl of standard HCG solution and sample were placed onto the circles of a dark background slide. Fifty µl of rabbit anti - β - HCG coated latex , after well mixing , was added onto the slide containing standard or sample and mixed well by a toothpick , and then rotated gentlely by hand. Agglutination occurred after 1 - 2 min. For semiquantitative assay , the sample would be serially diluted with phosphate saline buffer , pH 7.0.

II.4.4 Detection of HCG Activity by Commercial β - HCG EIA KIT. (Roche) (57)

Reagents

1. Beads coated with monoclonal anti - β - HCG (mouse) in 0.1 M Tris buffer
2. Monoclonal anti - β - HCG (mouse) - peroxidase conjugate > 2 u/ml in 0.1 M Tris buffer
3. Standard HCG : 0, 10, 25, 50, 100 and 200 mi.u./ml in normal human serum (1^{st} IRP for the immunoassay)
4. Control HCG : 54.2 mi.u./ml in normal human serum
5. Ortho - phenylenediamine (OPD) : 200 µmol/tablet
6. Subtrate buffer : 0.1 M citrate buffer , pH 5.2 , containing 6 mM H_2O_2
7. Sulfuric acid : 1.0 M

Procedure

The assay steps were carried out by following the method instruction of the Roche's β - HCG EIA KIT (57). Twenty ul of standard HCG or control HCG or each patient serum was placed into each tube and then 0.25 ml of the anti - β - HCG - peroxidase conjugate was added into all tubes. Monoclonal anti - β - HCG coated beads were added into all tubes and shaken gently , then incubated at 37 °C for 1 hr. After incubation , all tubes were washed three times with distilled water by Pastuer pipette attached to a vacuum pump. One tablet of OPD (200 µmol)

was dispensed in 5.0 ml of subtrate buffer and 0.25 ml of subtrate solution was added into all tubes and using a new tube as reagent blank control. The enzymatic reaction took place in dark condition at room temperature for 30 min , after then , 2.0 ml of sulfuric acid was added to stop enzymatic reaction. The O.D. at 492 nm of standards and samples were measured against reagent blank. A calibration curve is presented in Figure II.2 which will be used for the determination of HCG content in samples.

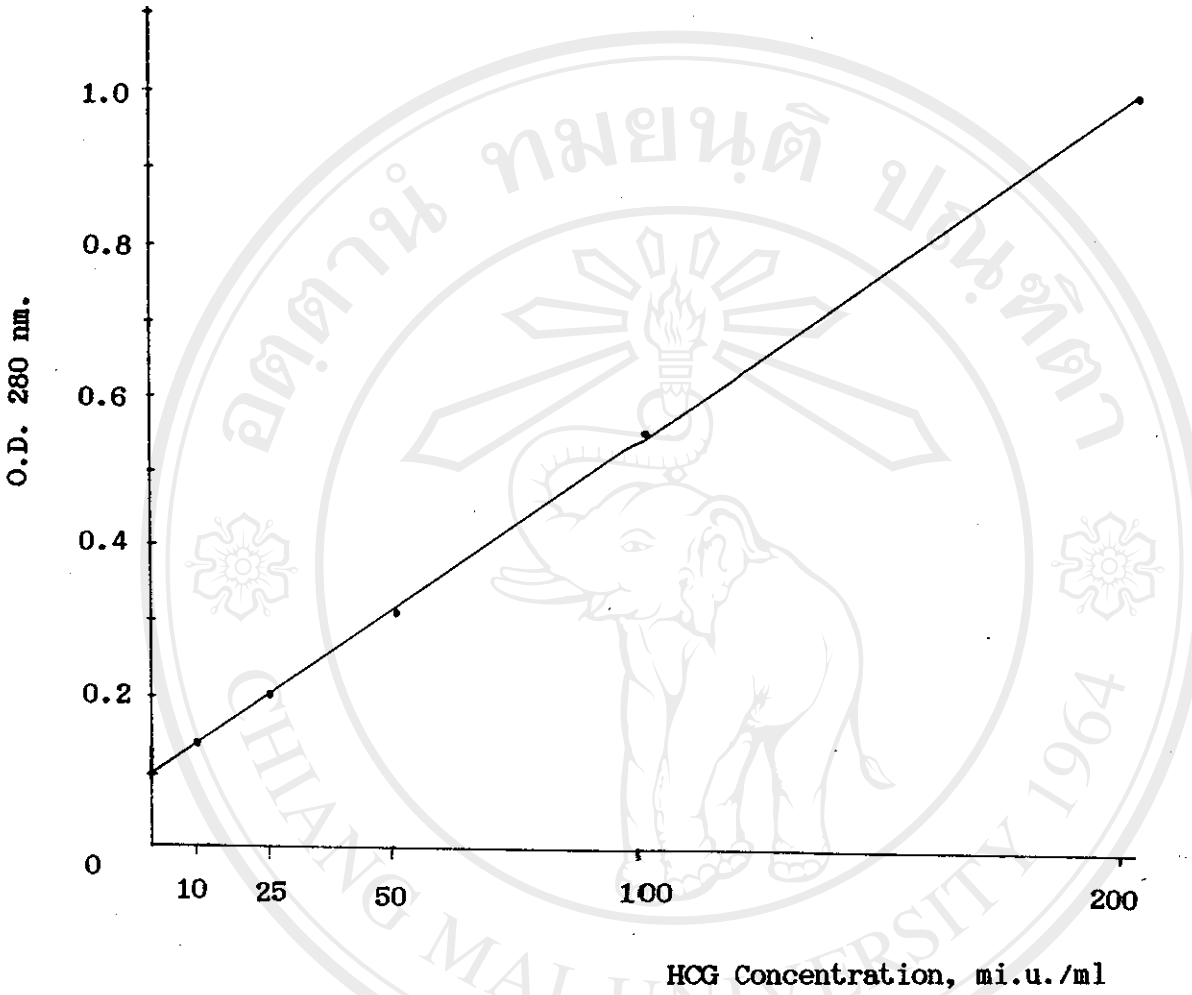


Figure II.2 Calibration Curve of Commercialβ - HCG ELISA (Roche)

II.4.5 Preparation of HCG - Sepharose 4 B and Normal Human Serum Protein - Sepharose 4 B. (63)

Reagents

1. Sepharose 4 B
2. Sodium carbonate buffer , pH 10.5 : 0.1 M
3. Sodium hydroxide : 0.1 M
4. Borate saline buffer , pH 8.3 : 0.15 M
5. Glycine : 1 M
6. Partially purified HCG
7. Normal human serum

Procudure

Five ml of packed Sepharose 4 B was washed three times with 0.01 M sodium carbonate buffer , pH 10.5 , and 0.1 M sodium carbonate buffer , pH 10.5 , was added to the final volume of 10.0 ml. pH meter was connected to the beaker containing Sepharose in a flow - hood. Five hundred mg of solid cyanogen bromide was added to the Sepharose , stirred and maintained the pH to 10.5 - 11.0 by adding 0.1 M sodium hydroxide until solid cyanogen bromide was completely disssolved and then kept in the flow - hood for 15 min. After then , cyanogen bromide - activated Sepharose was washed with 500 ml of 0.15 M borate saline buffer , pH 8.3. After washing , the cyanogen bromide - activated Sepharose gel was added in 5.0 ml of partially purified HCG (2.0 mg/ml) in 0.15 M borate saline buffer , pH 8.3 and mixed at room temperature

for 4 hr. After mixing , 1.0 ml of 1.0 M glycine was added to stop coupled reaction. Uncoupled protein was removed by centrifugation at 3000 g for 20 min and the amount of protein coupling to Sepharose was calculated. The HCG conjugated Sepharose 4B was washed with 0.02 M phosphate buffer , pH 7.0 , and stored in 0.02 M phosphate buffer , pH 7.0 , containing 0.1 % (w/v) sodium azide at 4 °C.

In case of the preparation for normal human serum protein - Sepharose 4 B , all procedures were principally the same as described above , but only in the coupling process , HCG was replaced by 10 mg/ml of pooled normal human serum protein.

II.4.6 Analysis of Anti - β - HCG Titer by Ouchterlony's Method (64)

Reagents

1. Agarose
2. Phosphate buffer , pH 7.0 : 0.05 M
3. Highly purified HCG : 500 i.u./ml

Procedure

One g of agarose in 100 ml of 0.05 M phosphate buffer, pH 7.0 was heated at 100° C to dissolve all the powder. Three ml of the melted agarose were poured onto a agarose - precoated microscopic slide (2.5 x 7.5 cm) standing on a horizontal surface and allowed to settle at room temperature. After setting , the agarose slide was punched six outer and one central wells by a gel puncher as shown in Figure III.8. Each well was 3 mm in diameter and distance between well to well was 10 mm , then the agarose plugs were evacuated by a Pastuer pipette attached to vac - cuum pump. Ten µl of 500 i.u/ml of highly purified HCG in phosphate saline buffer , pH 7.0 , was filled up into the central well and also 10 ul of two - fold diluted anti - β - HCG to be tested in phosphate saline buffer , pH 7.0 , were filled up into the series of outer wells. Then , the agarose slide was kept horizontal in a moistured chamber at 37°C , overnight. Precipitin lines would be visible as white cloudy lines in the gel. For permanent preparation , the agarose slide was immersed in normal saline for 2 days and in distilled water for 4 hr, respectively. The slide was dried at 70°C for 2 hr before staining with Coomassie blue.

II.4.7 Detection of Cross - Reactive Contaminant in Anti - B - HCG Preparation by Immunoelectrophoresis. (65)

Reagents

1. Barbitone buffer, pH 8.2 : 0.08 M
2. Agarose
3. Highly purified HCG : 500 i.u/ml
4. Normal human serum

Procedure

Three ml of 1 % melted agarose in 0.08 M barbitone buffer, pH 8.2 were poured onto a agarose - precoated microscopic slide (2.5 x 7.5 cm) standing on a horizontal surface and allowed to settle at room temperature. After setting , the agarose slide was punched a pair of wells (3 mm in diameter of well and 1.5 cm apart) at the middle of slide as shown in Figure III.10. Then , the plugs of wells were evacuated by Pastuer pipette attached to vacuum pump. Ten µl of 500 i.u/ml of highly purified HCG in phosphate saline buffer , pH 7.0 , containing 0.1 % (w/v) Bromophenol blue and 10 µl of normal human serum were filled up into each well. After applying , the agarose slide was placed in electrophoretic tank and the slide of agarose was directly connected with the wicks on the support bridges. A constant current of 5 mA was applied until the Bromophenol blue marker had migrated to within 0.5 cm of the anode wick, and then the agarose slide was taken off from electrophoretic tank. The templat block was placed over the agarose

slide and cut a trough of 0.2 x 6 cm with bladed knife at the middle between the pair of wells of the electrophoretic - separated agarose slide as shown in Figure III.10. The trough was filled up with rabbit - anti - β - HCG serum and the agarose slide was maintained in a moistured chamber at 37 °C , overnight. Precipitin lines were observed visually against dark background. For permanent preparation , the agarose slide was bathed for 2 days with normal saline and with distilled water for 4 hr and then dried at 70 °C for 2 hr before staining with Coomassie blue.

II.5 Experimental Methods.

II.5.1 Isolation and Purification of HCG and β - HCG Subunit. (19)

II.5.1.a Crude Precipitation From Urine.

Urine was collected from choriocarcinoma patients. All urinary samples had more than 100 i.u of HCG per ml detected by HCG - agglutination assay. Glacial acetic acid was used as a preservative at a concentration of 10 ml/L of urine. Choriocarcinoma urine (2 - 3 L) was acidified to approximated pH 3 with glacial acetic acid. Since isoelectric pH of HCG was about 3 , HCG precipitate was therefore expected. (8) While stirring , solid ammonium sulphate was slowly added. Until about 670 gm of ammonium sulphate per L of urine were totally added. Urinary pritein precipitate was collected by centrifugation at 5,000 g for 20 min and the supernatant was discared. The precipitate was dissolved with 50 ml. of 0.01 M phosphate buffer , pH 7.0 , and dialysed against 1 L of distilled water , three changes , at 4 °C. Undissolved precipitate was removed by centrifugation at the same speed and time. Brown - coloured crude HCG solution obtained was immunologically detected by HCG - agglutination assay.

The crude HCG solution was further precipitated with ethanol to remove brown - coloured substances. While stirring , cold absolute ethanol was slowly added to 80 % final concentration , then acidified to approximate pH 3 with formic acid and kept at 4 °C, overnight. Protein precipitate was collected by centrifugation at 10,000 g for 30 min , and

brown - coloured supernatant was discarded. The precipitate was dissolved with 50 ml of 0.01 M phosphate buffer , pH 7.5 , and dialysed against 1 L of 0.005 M phosphate buffer, pH 7.5 , Three changes, at 4°C. Undissolved precipitate was removed by centrifugation at the same speed and time and pale - yellow crude HCG solution was immunologically detected. Crude HCG solution was readily isolated by ion - exchange chromatography in the following step.

II.5.1.b DEAE - Sephadex A.50 Chromatography.

Five gm of DEAE - Sephadex A.50 was suspended in 0.005 M phosphate buffer , pH 7.5 , for 12 hr. The gel slurry was packed in a 2.5 x 15 cm column. The column was pre - equilibrated with 0.005 M phosphate buffer , pH 7.5 , at least 1 L. Crude HCG solution was adjusted pH to 7.5 with 0.1 M sodium hydroxide and applied on the column. The flow rate was calibrated at 1 ml/min and 8 – ml fractions were collected. The column was equilibrated with 0.01 M phosphate buffer , pH 6.6 , until eluated fraction had zero at O.D. 280 nm. After then, concave gradient of sodium chloride was used to elute the proteins adsorbed column at a flow rate of 1 ml/min. Sodium chloride concave gradient made from three chambered varygrad (66), each of which contained 500 ml of the following buffers , first , 0.01 M phosphate buffer , pH 6.6 second , 0.05 M sodium chloride , 0.02 M phosphate buffer , pH 6.6 , and the third , 0.25 M sodium chloride , 0.03 M phosphate buffer , pH 6.6 , and the buffers in the first and second

chambers were stirred all the time. Optical densities of eluated fractions collected were measured at 280 nm and detected of HCG activity by agglutination assay. Fractions with more than 2,000 i.u/ml were pooled and dialysed against distilled water at 4 °C and lyophilized. The lyophilizate was dissolved with 2 ml of 0.05 M phosphate buffer , pH 6.6 and assayed for immunological activity of HCG. This partially purified HCG was used in both the conjugation with Sepharose 4 B and in further purification.

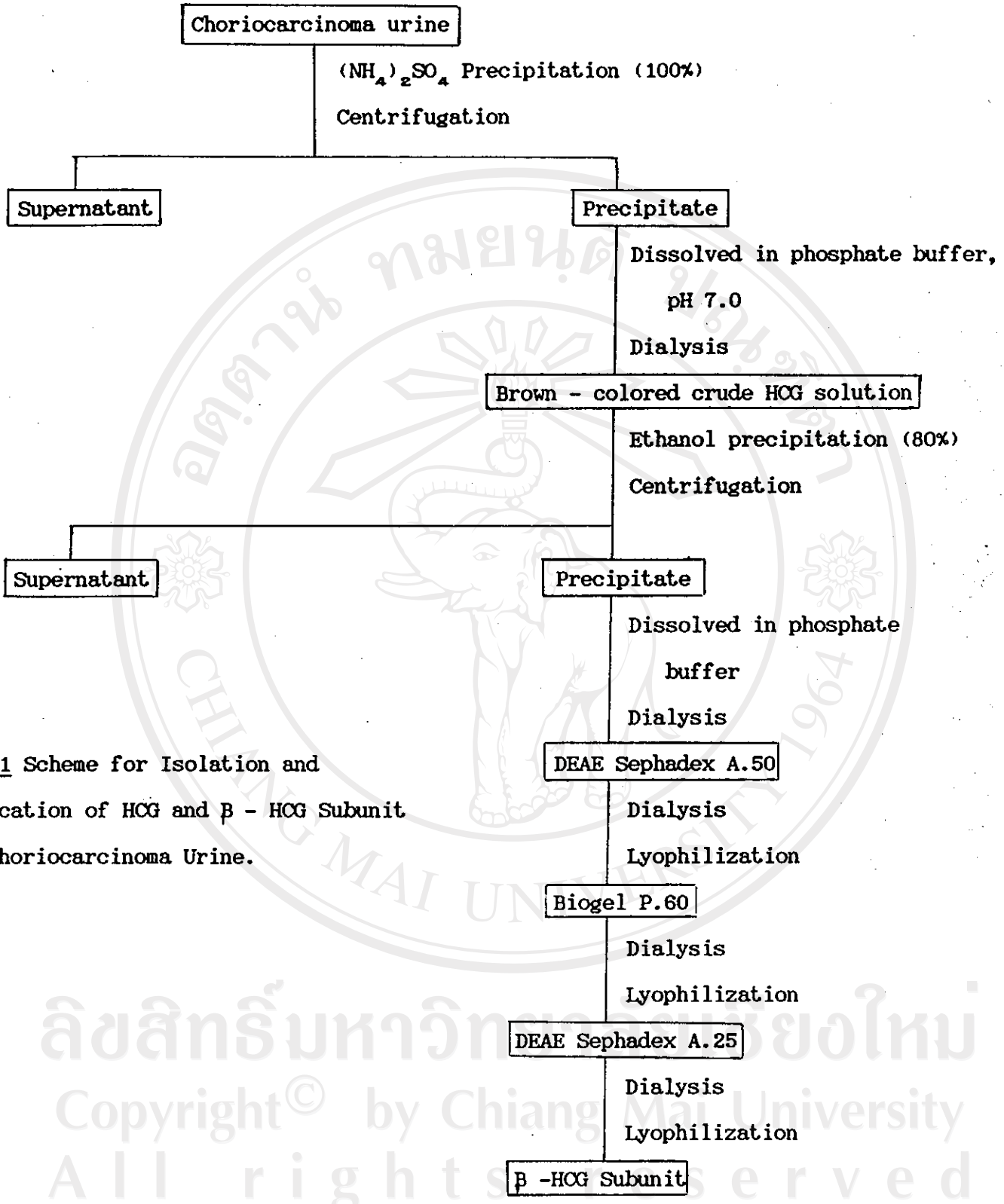
II.5.1.c Biogel P-60 Chromatography.

Biogel P-60 was suspended in 0.05 M phosphate buffer , pH 6.6 , and settled at room temperature for 72 hr. The gel was completely degassed and then packed in a 2.0 x 100 cm column. The column was equilibrated with 0.05 M phosphate buffer , pH 6.6 , at least two tatal volumes. The flow rate was calibrated at 6.0 ml/hr. Partially purified HCG was applied on the column and 4-ml fractions were collected. Optical densities at 280 nm of eluated fractions were measured. Fractions containing HCG more than 2,000 i.u/ml were pooled , then dialysed against distilled water at 4 °C and lyophilized. The lyophilizate was dissolved in 2 ml of distilled water and immunologically assayed for HCG by both agglutination and ELISA methods. Its purity was analysed by native and SDS - polyacrylamide gel electrophoresis. If HCG obtained was pure enough ,its specific activity as high as about 5,400 i.u/mg protein (2^{nd} I.S for the immunoassay) or 12,000 i.u/mg of protein (1^{st} I.R.P

for the immunoassay), it would be ready for subunit isolation in the next step.

II.5.1.d DEAE Sephadex A.25 Chromatography.

Two gm of DEAE - Sephadex A.25 was suspended and settled in freshly prepared 8 M urea , 0.03 M glycine , pH 7.5 , for 12 hr at room temperature. The gel was packed in a 1 x 12 cm column. The column was equilibrated with freshly prepared 8 M urea , 0.03 M glycine , pH 7.5 at a flow rate of 8 ml/hr. Twenty mg of highly purified HCG from step II.5.1.c was dissolved in 5 ml of 10 M urea adjusted pH to 4.5 with 0.1 M hydrochloric acid and incubated at 40 °C for 1 hr. Then, 1 ml of 0.03 M glycine was added and the mixture was adjusted to pH 7.5 with 0.1 M sodium hydroxide and applied on the column. Initial elution was performed with the above equilibrating buffer. Four - ml fractions were collected. The column was eluted until the last fraction contained no more protein. Then , the column was stepwise eluted with 8 M urea ,1.0 M NaCl , 0.2 M glycine , pH 7.5 . The second protein peak appearred. Fractions under each protein peck were pooled, acidified , dialysed against 1 % acetic acid and against distilled water , repectively before lyophilization. The second protein peak contained β - HCG subunit was analysed for its purity by SDS - polyacrylamide gel electrophoresis.



ırt II.1 Scheme for Isolation and Purification of HCG and β - HCG Subunit from Choriocarcinoma Urine.

II.5.2 Production and Preparation of Rabbit Anti - β - HCG

II.5.2.a Production of Rabbit Anti - β - HCG. (68)

Fifty ug of purified β - HCG subunit was dissolved in 1 ml of normal saline solution and 1 ml of completed Freund's adjuvant was added. The solution and oil were mixed vigorously until white - cream emulsion would be formed and ready for the injection. One ml syringe and needle No.27 was used to inject the emulsion into a 3 - 4 month old New Zealand white rabbit subcutaneously for 20 - 30 sites. Four weeks later , the immunized rabbit was booster - injected with the same dose of β - HCG subunit , but incompleted Freund's adjuvant was used instead of completed Freund's adjuvant. Two weeks after booster - injection , the rabbit was bled 1 ml of blood and rabbit serum was detected for anti - B - HCG titer by Ouchterlony's method. When the titer was 1 : 4 , the rabbit would be bled for 40 ml of blood. The blood was set at room temperature for 2 - 3 hr for clotting , and then centrifuged at 3,000 g for 20 min. Rabbit serum was collected with a capillary pipette. The serum would be used for the preparation of IgG.

II.5.2.b Preparation of Rabbit IgG. (55)

A half volume of saturated ammonium sulfate solution (13 ml) was slowly added into the rabbit serum (26 ml) while stirring for 33 % ammonium sulfate saturation. IgG was precipitated and kept at 4 °C, overnight. The precipitated IgG was collected by centrifugation at 5,000 g for 30 min and the supernatant was discarded. The IgG was suspended

with 30 ml of ammonium sulfate solution (33 % saturation) and centrifuged again under the same condition. The supernatant was discarded. The IgG precipitate was dissolved in 10 ml of 0.02 M phosphate buffer , pH 7.0 , and dialysed against 1 L of 0.02 M phosphate buffer , pH 7.0 , three changes , at 4 °C for 24 hr. Anti - β - HCG titer in IgG preparation was detected by Ouchterlony's method. This preparation was further purified by affinity chromatography.

II.5.2.c Affinity Chromatography (55)

HCG - Sepharose 4 B , as previously prepared from partially purified HCG (Section II.4.5) was suspended in 0.02 M phosphate buffer , pH 7.0 , and packed in a 1 x 15 cm column. The column was pre - equilibrated with 0.02 M phosphate buffer , pH 7.0. The rabbit IgG preparation was applied on the column. First elution was performed by passing equilibrating buffer at a flow rate of 2 ml/hr and 4 - ml fractions were collected until the last fraction contained no more protein. The second elution was done by passing 0.01 M glycine - HCl buffer , pH 2.5 , at the fasted flow rate and the eluted fractions were neutralized to pH 8.0 with 0.1 M sodium hydroxide. Protein containing fractions under the second peak were pooled , dialysed against distilled water at 4 °C for 24 hr and lyophilized. The lyophilizate was dissolved in 1 ml of 0.02 M phosphate buffer , pH 7.0 , and detected for anti - β - HCG titer by Ouchterlony's method. Any cross - reaction to normal human serum protein was also carried out by immunoelectrophoresis. If anti - β - HCG showed

no cross - reaction to normal human serum protein , the anti - β - HCG preparation was adsorbed undetectable cross - reactive antibody by normal human serum protein - Sepharose 4 B chromatography.

Normal human serum protein – Sepharose 4 B as previously prepared from pooled normal human serum protein (Section II.4.5) was suspended in 0.02 M phosphate buffer , pH 7.0 and packed in a 1 x 10 cm column. Rabbit anti - β - HCG prepared from HCG - Sepharose 4 B chromatography was applied on the column. All procedure was carried out the same as HCG - Sepharose 4B chromatography but protein containing fractions under the first peak were pooled , dialysed and lyophilized. Glycine - HCl buffer , pH 2.5 eluted protein containing fractions under the second peak were discarded. The lyophilizate was dissolved in 1 ml of 0.01 M sodium carbonate buffer pH 8.3 and detected for anti - β - HCG titer by Ouchterlony's method. Cross - reactive antibody to normal human serumprotein was detected by immunueletrophorosis. If anti - β - HCG showed no cross - reaction to normal human serum protein , anti - β - HCG preparation was used in experimental enzyme immunoassay.

II.5.3 Preparation and Isolation of Anti - β - HCG - Horseradish Peroxidase Conjugate. (69)

Three mg of horseradish peroxidase (HRP) , R.Z. 2.0 , were dissolved in 1.0 ml of 0.3 M sodium carbonate buffer,pH 8.1 and then 0.1 ml of 1.0 % (v/v) 1,4 - fluorodinitrobenzene (FDNB) in absolute ethanol was added and mixed gently at room temperature for 1 hr. After mixing , 1.0 ml of 0.08 M sodium periodate was added in FDNB - HRP solution and mixed at room temperature for 30 min , the solution was green - yellow color. After then , 1.0 ml of 0.16 M glycerol was added to destroy excess periodate , stirred at room temperature for 1 hr and dialysed against 1 L of 0.01 M sodium carbonate buffer , pH 9.5 , three changes , at 4 °C for 24 hr. After dialysis , 3.0 mg of anti - β - HCG in 0.5 ml of 0.01 M sodium carbonate buffer , pH 9.5 , was added to HRP - aldehyde solution and mixed gently at room temperature for 3 hr. After mixing , 2.0 mg of solid sodium borohydride was added and kept at 4 °C for 6 hr. After then , the conjugate mixture was dialysed against 1 L of phosphate saline buffer , pH 7.0 , three changes , at 4 °C for 24 hr. After dialysis , the conjugate mixture was applied on the 1 x 75 cm column of Sephadex G.150 pre - equilibrated with phosphate saline buffer , pH 7.0. A flow rate was calibrated at 4.0 ml per hr and 2 - ml fractions were collected. Optical densities at 280 and 403 nm of eluated fractions were measured. The fractions under the major peak were pooled and kept at -20 °C in an equal volume of glycerol.

II.5.4 Preparation of Reference Standard HCG Using in Enzyme Immunoassay.

Highly purified HCG as previously prepared in section II.5.1 was dissolved in pooled normal human serum to 1,000 mi.u./ml final concentration (1st I.R.P for the immunoassay) detected by commercial β - HCG EIA (Roche). Then , the stock standard HCG was diluted with pooled normal human serum containing 0.1 % merthiotate to 200, 100, 50, 25 and 12.5 mi.u./ml , respectively and stored at 4°C until use. Pooled normal human serum was used as null reference standard. All reference standard were standardized again by commercial β - HCG EIA. (Roche)

II.5.5 Development of Enzyme Immunoassay for HCG

The two - step sandwich enzyme immunoassay of HCG had been employed in 12 x 75 mm polystyrene tubes. The solid phase , anti - β - HCG coated polystyrene tubes were prepared by physical adsorption in alkaline condition of carbonate buffer , pH 9.5 , at room temperature. The tubes were previously soaked in 95 % ethanol for 1 hr and then washed with distilled water before coating. (70) After coating process , bovine serum albumin was used as a non - specific protein for blocking. Reaction between antibody with antigen and chromogenic development was done in 1.0 ml volume. The first reaction , solid phase anti - β - HCG with HCG was performed by adding 0.2 ml of serum sample or standard HCG prepared in normal human serum (0, 12.5, 25, 50, 100 and 200 mi.u/ml) and 0.8 ml of phosphate saline buffer, pH 7.0 , containing 0.05 % Tween 20 (v/v) and incubating at 37°C for suitable periods. After the first reaction , the tube were washed three times with phosphate saline buffer , pH 7.0 , containing 0.05 % Tween 20 (v/v) (washing buffer). The second reaction , between solid phase anti - β - HCG bound HCG and anti - β - HCG - horseradish peroxidase (HRP) conjugate , was also performed by adding 1.0 ml of suitable dilution of anti - β - HCG - HRP conjugate in phosphate saline buffer , pH 7.0 , containing 0.05 % Tween 20 (v/v) and 12.5 % chicken plasma (v/v). The tubes were incubated at 37°C for suitable periods and then washed three times with washing buffer. Chromogenicity was developed by adding 1.0 ml of 0.2 % ortho -

phenylenediamine (OPD) in 0.1 M citrate buffer pH 5.4 containing 10 mM H_2O_2 to all tubes using a new tube as reagent blank control. The enzymatic reaction took place in dark condition for 30 min at room temperature (71) after then , 1.5 ml of 1.0 M sulfuric acid containing 0.01 M sodium sulfite was added to stop enzymatic reaction. The O.D. at 492 nm was measured by a LKB spectrophotometer adjusted at zero with a reagent blank.

Different conditions for enzyme immunoassay of HCG e.g. dilution of anti - B - HCG for coating , dilution of anti - β - HCG - HRP conjugate , incubation periods for the first and second reaction , effect of protein added to the conjugate , precision , sensitivity , analytical recovery and stability of the reagents have been investigated and reported as the results in Chapter III.

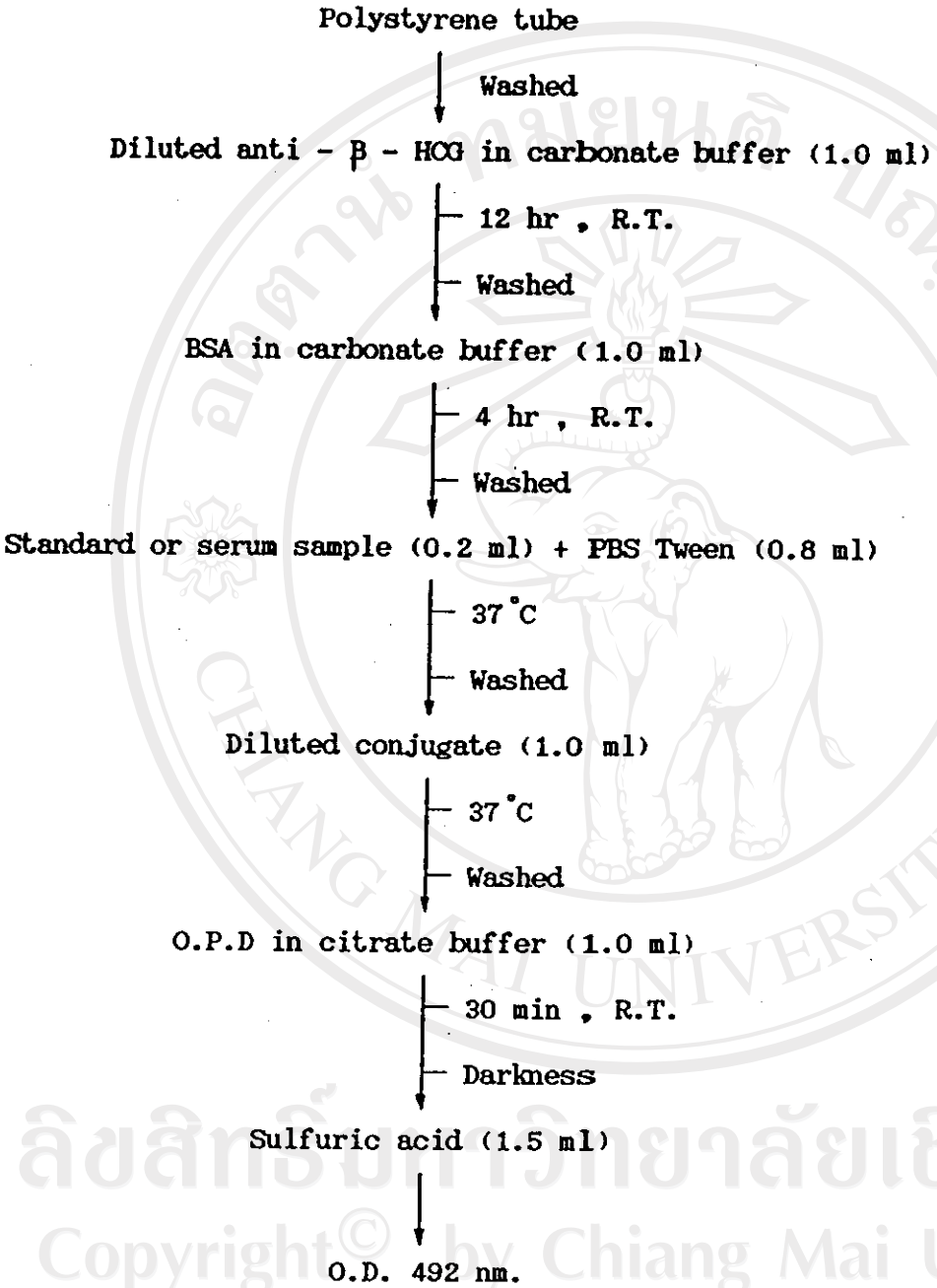


Chart II.2 Procedure Steps of Enzyme Immunoassay for HCG.