IV Discussion

IV.1 Isolation and Purification of HCG and β - HCG Subunit from Choriocarcinoma Urine

The first objective of this study was the preparation of purified β - HCG subunit to be used as immunogen for rabbit anti - β - HCG production. Ideally, the antigen must be not contaminated by other human proteins but retain immunological activity. It was also necessary to prepare i.e. partially purified HCG used in the conjugation for HCG - Sepharose 4B, and highly purified HCG as a reference standard using in β - HCG enzyme immunoassay (β - HCG EIA)

The isolation and purification of HCG from pooled choriocarcinoma urine were step - wise performed by saturated ammonium sulfate precipitation, ethanol precipitation, DEAE Sephadex A.50 and Biogel P - 60 chromatography, respectively. β - HCG Subuit was isolated from the HCG and purified by 8 M - urea DEAE Sephadex A.25 chromatograhpy.

The yield of crude HCG isolated from pooled choriocarcinoma urine by ammonium sulfate and ethanol precipitation was 42%. It was quite high as compared to 25% reported by Nishimura et al (23) who used kaolin - acetone extraction and ethanol precipitation. Commercial crude HCG powder was generally used as the starting material for purified HCG preparation and reported by several investigators.(19,24) They generally used ethanol precipitation in order to remove interferring substances. The preparation of crude HCG by ammonium sulfate precipitation in this study was economical and high reproducibility.

After DEAE Sephadex A.50 chromatography, the yield of partially purified HCG was 31% of starting urine and that of purified HCG after Biogel P-60 chromatography was 28%. These yields were markedly high compared to 21 % and 18%,respectively reported by Nishimura et al. (23)

The immunologically specific activity of highly purified HCG preparation was 9,250 i.u./mg protein (1^{st} I.R.P for immunoassay) quantitatively detected by β - HCG EIA (Roche) and the purification fold was increased 26 times of the starting urine. The specific activity was closed to that 9,750 i.u./mg protein reported by Nishimura et al (23) using DEAEcellulose and Sephadex G.100 chromatography for choriocarcinoma HCG purification (23). Chang et al (73) obtained purified pregnant HCG preparation with biologically specific activity of 11,000 i.u./mg protein (2^{nd} I.S. for bioassay, which is equivalent to 1^{st} I.R.P for the immunoassay) by only DEAE cellulose chromatography. Canfield et al (19) reported the biologically specific activity between 13,700 - 18,600 i.u./mg protein for their pregnant HCG preparation. Their purification procedure had been used in this thesis. However, the variation of specific activity of HCG reported was also due to the variation of biological assay methods. Several investigators established that the immunological activity of HCG from different sources were similar but the biological activity of choriocarcinoma HCG was very low, because its sialic acid content was absent. Thus, detection methods

based on immunological principle can give actual level of choriocarcinoma HCG. (23,24)

In this study, the latex agglutination assay was used in the semi - quantitative estimation of HCG in all the preparations. The cost of agglutination reagents was very low compared to commercial β - HCG EIA. The specific activity of purified HCG preparations were approximately 5,000 i.u./mg protein (2^{nd} I.S. for the immunoassay) estimated by the agglutination assay. Recently, very highly purified HCG with its immunologically specific activity of 5,400 i.u./mg protein (2^{nd} I.S. the for the immunoassay) or 12,00 i.u./mg protein (1^{st} I.R.P for the immunoassay) could be prepared.(23,44) Thus, the immunologically specific activity of the HCG preparation was so suitably high and used for β - HCG subunit preparation.

The immunologically specific activity of B - HCG subunit preparation was 14,350 i.u./mg protein (1^{st} I.R.P for the immunoassay) and approximately 7,500 i.u./mg protein (2^{nd} I.S. for the immunoassay). The high increase of specific activity of β - HCG subunit preparation indicated that α - HCG subunit was excluded from this preparation. By molar ratio, the specific activity of this β - HCG subunit preparation would theoretically increase twice, but it practically and approximately increased 1.5. Two possible ways would be hypothesized that the β - HCG subunit preparation lost its activity in the processes of subunits dissociation, purification and lyophilization or this preparation was con-

taminated with other proteins.

By native - polyacrylamide gel electrophorosis, the electrophoretic patterns of crude HCG after ammonium sulfate precipitation were similar to those of crude HCG after ethanol precipitation, since ethanol precipitation removed only non - protein contaminating substances. However, there was only one protein band in both electrophoretic patterns corresponding to that of standard HCG. HCG fractions collected from DEAE Sephadex A.50 chromatography showed one major bands corresponding to that of standard HCG and two other minor bands with their molecular weight less than HCG. Canfield et al (19) showed that some fractions collected after DEAE Sephadex A.50 chromatography showed only one major band of HCG. The protein contaminants in fractions collected from DEAE Sephadex A.50 suggested that there were contaminated protein contained in the collected fractions. After Biogel P - 60 chromatography, the purified HCG preparation showed one major band corresponding to standard HCG and a very small band of contanminating protein with molecular weight less than HCG. Canfield et al (19) and Nishimura et al (23) reported only one electrophoretic band of their highly purified HCG preparation in native - polyacrylamide gel electrophoresis after the steps of Biogel P-60 and Sephadex G.100 chromatography, respectively. In this study, Biogel P-60 chromatography excluded most of contaminting proteins. This HCG preparation was pure enough for the preparation of β - HCG subunit.

By SDS - polyacrylamide gel electrophoresis, the electrophoretic pattern of β - HCG subunit preparation showed only one band corresponding to the slower moving band of standard HCG (beta subunit). The electrophoretic patterns of α -subunit fraction, showed one major band corresponding to the faster moving band of standard HCG, and one contaminating minor band which molecular weight less than the alpha subunit. As expected, highly purified HCG gave two major bands of alpha and beta subunits in SDS - polyacrylamide gel electrophoresis corresponding to those of standard HCG. Although, the α - HCG subunit preparation was not completely purified , but the β - HCG subunit preparation was pure enough to be used as immunogen. The loss of β - HCG subunit activity in preparation could be due to the process of subunit isolation or dialysis or lypophilization.

IV.2 Production and Preparation of Rabbit Anti - β - HCG.

Afetr two weeks of booster injections, only three rabbits produced anti - β - HCG of 1 :4 titer detected by Ouchterlony's method, and four rabbits had 1 : 2 titer while the other four gave no response. Several investigators established that β - HCG subunit was not good immunogen since it contained a large amount of carbohydrate moiety (29 - 36 %) protecting antigenic determinants and its primary structure was related to mammal gonodotropic hormones. (8,74)

The rabbits produced 1 : 4 anti - β - HCG titer were bled and IgG was prepared by ammonium sulfate precipitation (33% saturation). The IgG yield was 96 % retained of total titer. By HCG - Sepharose affinity chromatography, it needed at least 3 hr for maximal reaction between anti - β - HCG and HCG - Sepharose. In the acid elution step, the flow rate must as fast as it would be, since the immunologlubulin was not stable in low pH solution. The yield of anti - β - HCG obtained in this step was 15 % from starting rabbit anti - β -HCG antiserum. Rabbit anti - β - HCG antiserum, rabit IgG and anti - β - HCG preparation did not show any cross - reaction to normal human serum proteins detected by immunoelectrophoresis due to the low sensitivity of dectection method. Undetectable cross - reactive antibody in anti - β - HCG preparation was removed by normal human serum proteins - Sepharose affinity chromatography. A small amount of protein in anti - β -HCG preparation adsorbed on the column, could be cross - reactive antibody or non - specific adsorbed

antibody. The yield of adsorped anti - β -HCG was 15 %. It was closed to 10 % that reported by Vaitukaitis et al. (40) However, the adsorbed anti - β -HCG preparation did not show any cross - reactivity to normal human serum protein detected by immunoelectrophoresis but it exhibited some cross - reaction to other gonadotropic hormanes, LH , FSH and TSH by enzyme immunoassay. Since, physiological concentration of serum LH, FSH and TSH is usually very low , therefore their cross - reactive antibodies could not be completely adsorbed on the affinity column.

Specific activity of the final anti - β - HCG preparation was 1111 $titer^{-1}$/mg protein which comparable to 1300 $titer^{-1}$/mg protein of standard anti - β - HCG (DAKO)

IV.3 Development of Enzyme Immunoassay for HCG

In this study, periodate oxidation method was performed for the preparation of anti - β - HCG - HRP conjugate. After Sephadex G.150 chromatography, approximate 27 % of total HRP activity was found to be conjugated with anti - β -HCG. The conjugated amount was low compared to 50 % reported by Zaitsu et al (56). Although, the Sephadex gel could not separate antibody - HRP conjugated from unconjugated antibody but Wilson and Nakane claimed that 99 % of antibody was labelled with HRP by their method.(69) By molar ratio, one mole of anti - β - HCG was conjugated with one of HRP in this conjugate preparation. Wilson and Nakane reported that antibody would loose or reduce its immunological activity ,if it was conjugated with more than 4 moles of HRP. (69)

A number of solid phases such as polystyrene, polypropylene , polyvinyl chloride and silicone rubber were introduced for antibody support. However, polystyrene is popularly used, since antibody molecule can be adsorbed physically onto the polymer and difficultly dissociated from this solid phase. Polypropylene and polyvinyl chloride cannot support antibody for a long time. Silicone rubber is similar to polystyrene but difficult to be uniformly coated use. Plastic beads and microplate are commonly use in a small volume EIA technique. Strips can be conveniently used in qualitative EIA technique. Test tubes should be suitable for a large volume of samples in order to increase the sensitivity. In this study, 12 x 75 mm. polystyrene tubes were selected for anti - β -

HCG support.

Antibody coating was taken place in carbonate buffer, pH 9.5, since this alkaline condition was appropriated for physical adsorption with out loss of antibody activity. Bovine serum albumin was usually employed as non - specific protein for blocking in order to protect dissociation of coated antibody and non - specific binding of antibody - enzyme conjugate. Incubation times of 12 and 4 hr for coating and blocking process, respectively were usually extra. (70)

In this study, the developed EIA technique for HCG was done in a 1 ml volume. The dilution of anti - β - HCG at 1:500 which was equivalent to 1μg/ml was found to be appropriated for coating. Since its dose-response curve was not different from those of the 1:250 and 1:125 dilutions, while the 1:1000 dilution showed significant decrease of dose - response curve. Concentration of antibody for coating were generally 1 - 10 μg/ml reported by several investigators. (50,51,70)

The conjugate was dissolved in phosphate saline buffer, pH 7.0, containing 12.5% chicken serum and 0.05% Tween 20 in order to protect non-specific binding to the solid phase. This concentration of chicken plasma was commonly used in EIA technique. However, when the concentration of chicken serum in the conjugate solution was decreased, the excess conjugate was non-specifically bound to the solid phase and represented by the increase of the O.D. value of background as shown in Figure III.14.

The criteria for selection of appropriated conjugate dilution used in EIA technique was that such conjugate dilution would give a low O.D. value of HCG - free serum or null standard, and highly different O.D. values between two dajacent HCG concentration. The 1:200 dilution of conjugate was finally chosen and used in the developed β - HCG EIA technique.

A kinetic study of incubation period for the first reaction, at low and medium HCG concentrations (12.5 and 50 miu/ml) were carried out at 2 and 4 hr , respectively for full binding between solid phase anti - β - HCG and HCG, while higher HCG concentration (200 miu/ml) needed to be incubated longer than 6 hr. For the second reaction, 1 and 2 hr were needed for full binding of the low and medium HCG concentrations, while high HCG concentration needed longer than 4 hr. Theoretically, calibration curve of this EIA technique was linear, when the incubation period for the first and second reaction were maintained longer than 6 and 4 hr, respectively. However, these incubation periods were too long and could not be practical for one day analysis. Thus, the incubation periods of 4 and 2 hr were chosen and a satisfactory calibration curve was obtained. Although, the curve was not linear but it gave accepted values of precision, recovery and accuracy. Several investigators reported different incubation periods for the two - step sandwich β - HCG EIA. Zaitsu et al (56) used 2 and 2 hr for the first and second reaction, respectively at 30 °C. Sekiya et al (55,b) reported 3 hr at 30 °C

and 12 hr at 4° C for the first and second reactions, respectively. The one - step β - HCG EIA (Roche) needed only 1 hr for antigen - antibody reaction.(57)

The O.D. value of null standard was usually between 0.05 - 0.1 due to nonspecific binding of the conjugate to solid phase and autooxidation of chromogenic subtrate, H_2O_2 and OPD. The sensitivity of this developed EIA method was 4 mi.u./ml which was lower than that of the Roche EIA kit, 1.7 mi.u./ml. Sekiya et al (55,b), Zaitsu et al (56) and Wada et al (50) reported that the sensitivity of their two - site sandwich β - HCG EIA rabbit anti - β - HCG were 6, 3 and 5 mi.u./ml, respectively.

The precisions of both within and between assays of the developed EIA technique were well - accepted. Serum samples with high HCG levels at an appropriated dilution with pooled normal human serum were assayed, small variation of the results were observed.

It was found that, the developed β - HCG EIA showed 24 and 15% cross - reactivity with LH and FSH at the concentration of 100 and 75 mi.u./ml, respectively. These cross - reactivity were noticedly high.

Therefore, the lower physiological concentration of serum LH and FSH which are usually 25 and 10 mi.u./ml, respectively should not significantly interfere in the HCG assay. However, somewhat high serum concentration of LH and FSH of women within the stage of ovulation, 50 and 20 mi.u./ml, respectively, may affect the HCG assay.

Wada et al (50) and Sekiya et al (55,b) reported 8.3 and 4 %, respectively for the cross - reactivity of LH on their sandwich β - HCG EIA using rabbit anti - β - HCG. Sekiya et al (54,a) reported 40% that of LH on their sandwich HCG EIA using rabbit anti - intact - HCG. Since, the primary structure of β - HCG subunit is 80% homology to that of LH, thus polyclonal anti - β - HCG, especially anti - intact - HCG cross - reacts highly to LH. Variations of the cross - reacting anti - β - HCG are also dependent on individual rabbits.(75)

TSH was not tested for cross - reaction but expected that its cross - ractivity was closed to that of FSH. Since, primary structure of beta subunits of TSH and FSH are nearly homological to that of HCG.

Due to the cross - reaction effect of the anti - β - HCG using in developed β - HCG EIA, mean serum HCG in normal subjects (5.0 and 9.5 mi.u./ml for men and women, respectively was higher than that detected by the Roche kit. (less than 1.7 mi.u./ml for both men and women). By the developed β - HCG EIA, the mean serum HCG in women was higher than in men. Distribution of serum HCG in women ranged <4 - 20 mi.u./ml while those of men were <4 - 10 mi.u./ml. Some normal women in ovulated stage had their serum HCG above 10 mi.u./ml.

Good correlations between the developed β - HCG EIA and the reference method (Roche kit) could be demonstrated with the regression equation $Y = 1.01X + 3.34$ and the correlation coefficient (r) = 0.945. Actually, in patients with hydatidiform mole, choriocarcinoma, ectopic

or normal pregnancy, their high HCG levels may regulatively inhibit the secretion of LH and FSH, thus their serum LH and FSH are very low or undetectable (76). Therefore, serum β - HCG can be used to evaluate in these patients for clinical diagnosis, monitoring and prognosis.

Comparison of characteristics and procedure steps performed both in the developed β - HCG EIA and commercial β - HCG EIA (Roche) are present in Table IV.1.

Table IV.1 Comparison of Characteristics and Procedure Steps Performed in Both the Developed and Commercial β - HCG EIA Kits.

Characteristics	Developed kit	Commercial kit(Roche)
Solid phase	Tube	Bead
Antibody	Rabbit anti-β-HCG	Monoclonal anti-β-HCG
Assay volume (µl)	1,000	270
Sample volume (µl)	200	20
Incubation period (hr)		
first reaction	4	1
second reaction	2	-
Washing period (min)	20	20
Chromogenic - developing period (min)	30	30
Time used for overall procedure (hr)	8	2
Sensitivity (mi.u./ml)	4	1.7
Cross-reactivity (%)		
LH	24	<1
FSH	15	<1
Price per test (Baht)	5	100

IV.4 Further Study

Several investigations for better development of the β - HCG EIA test should be further studied. Since high cross - reaction effect of LH and FSH was observed in this developed β - HCG EIA, this effect could be minimized by replacing rabbit anti – β - HCG used in the enzyme conjugate with other highly β - HCG subunit specific antibody such as anti - C - terminal peptide of β - HCG subunit and commercial monoclonal anti - β - HCG. The local - made anti - β - HCG can be effectively used for solid - phase coating.

Incubation periods for 6 hr may be time - consuming, the reduction of the incubation periods should be further studied by using higher concentrated or highly potent anti - β - HCG for tube coating. Moreover, the two - step assay may be improved to one - step technique.

Other solid phases such as beads, microplates and strips can replace the test tubes, since polystyrene tubes are not so economical in term of its high cost and much volume of reagents used.

The solid - phase immobilized anti - β - HCG may be experimentally prepared by covalent linkage for longer stability, since the stability of physically adsorbed antibody on solid phase is usually less than 2 weeks.

To develope the specificity of the method for the identification and quantitation of choriocarcinoma HCG and / or prenant and hydatidiform mole HCG , it is suggested that sialic acid - specific lectins such

as limulin and wheat germ agglutinin should be used to replace anti - β - HCG immobilized the solid phase. Before EIA test the malignant HCG in serum samples could be separated and eluted out .

Finally, in addition to HCG, enzyme immunoassay for other three gonadotropic hormones LH, FSH and TSH can be routinely set up by coating anti - alpha - subunit as coparticipated solid - phase, β - subunit specific antibody for each hormone will be prepared for the enzyme conjugate which can detect qualitatively and quantitatively the corresponding hormones.

If the above suggestions should be achieved, the home - made EIA kits for important gonadotropic hormones would be certainly clinically valuable and economically beneficial.