A New Radioimmunoassay for HCG Using Polyethylene Glycol

Fumihisa MIYAUCHI, Takehisa ITO, Yuichi KIDO and Tadashi TORIGOE

Department of Obstetrics and Gynecology, Yamaguchi University School of Medicine, Ube, Yamaguchi, Japan

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INTRODUCTION

Separation of free from antibody bound human chorionic gonadotropin (hCG) has been accomplished by several techniques of radioimmunoassay; the double antibody method[1-5], the coated charcoal method[6,7], the ammonium sulphate salting-out method[8], the alcohol precipitation method[9,10], and the dioxan precipitation method[11]. We have previously reported a double antibody radioimmunoassay method for hCG[5]. In this paper a new method using an aqueous polymer two-phase system for measurement of hCG is discussed. The procedure is a modification of the method reported by Desbuquois and Aurbach[12].

MATERIALS AND METHOD

Chemicals Purified hCG and guinea pig antiserum to hCG were prepared as previously described[5]. We obtained I^{125} from New England Nuclear, Boston; polyethylene glycol 6000 from Wako Pure Chemical Industries, LTD., Tokyo; gamma-globulin named human immune serum globulin, which contained aminoacetic acid 2.25 w/v% and thimerosal 0.01 w/v%, from Green Cross Co., Osaka; and crystalline bovine serum albumin (Fraction V) from Katayama Chemical Industries Co., LTD., Osaka.

Radioimmunoassay Purified hCG was labeled with I^{125} according to the method of Hunter and Greenwood[13]. The iodination mixtures were freed of the "damaged" component and unreated iodine according to a previously described procedure[5]. The labeled hCG was diluted by a buffer containing bovine serum albumine to prevent absorption onto glassware.

The labeled hormone was incubated at 4°C, 1) without antibody, 2)

with excess antibody to bind most of tracer, or 3) with the antibody diluted to bind part (usually 30 to 50%) of the tracer and increasing amounts of unlabeled hCG or unknown serum. The total volume of the incubation mixture was adjusted to 0.9 ml with a buffer containing 0.1% bovine serum albumin. After incubation at 4°C for 2 days, 0.1 ml of gamma globulin, which is known to be precipitated by polyethylene glycol[14], was added as a carrier protein immediatly prior to the separation step. Then, 1 ml of cold polyethylene glycol solution was added to the incubation mixture. The contents of the tubes were mixed with a Vortex mixer and centrifuged at 2600 rpm for 45 minutes in a refrigerated centrifuge. The radioactivities of the incubation mixture (T) and the sediment (B) were determined with a well-type automatic gamma counter (Aloka TDC-501).

RESULTS

Solubility of Bound or Free Labeled HCG in Polyethylene Glycol Our experiments were intended to determine the conditions for the optimal separation of free from antibody bound hCG.

The effect of the concentration of polyethylene glycol on the solubility of the labeled hCG is presented graphically in Fig. 1. As the concentration of polyethylene glycol was increased, free hCG coprecipit-

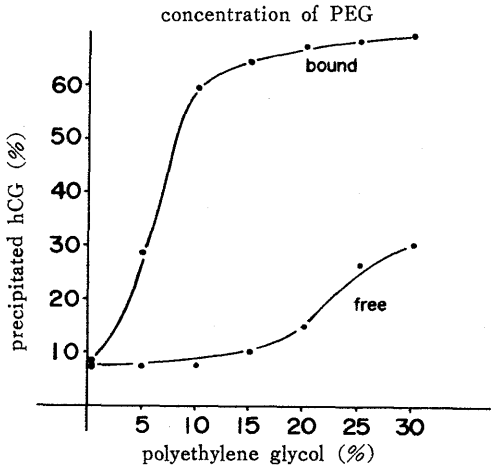

Fig. 1. Solubility of I^{125} labeled hCG as a function of polyethylene glycol concentration. Solubility was determined in phosphate beffer, 0.1 M, pH 7.4; anti-hCG serum 1 : 1,000 ; gamma globulin 0.15 mg/ml.

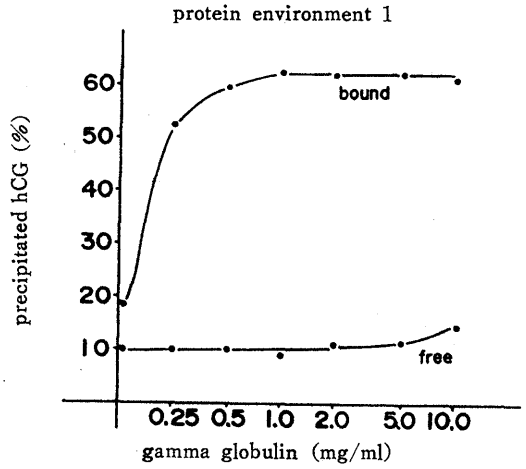

Fig. 2. Solubility of I^{125} labeled hCG as a function of concentration of gamma globulin. Solubility was determined in phosphate buffer, 0.1 M, pH 7.4 ; anti-hCG serum 1 : 1,000 ; polyethylene glycol concentration 12.5%.

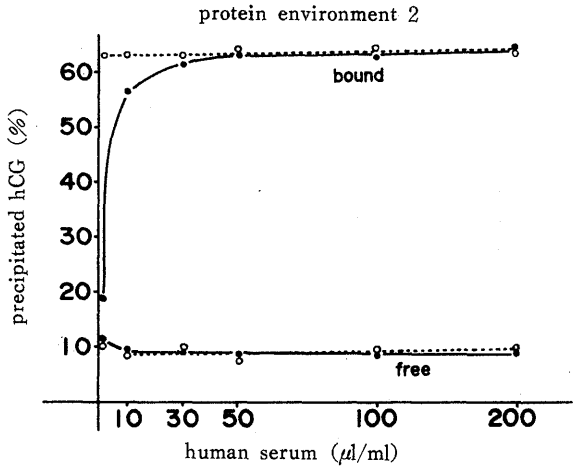

Fig. 3. Solubility of I^{125} labeled hCG as a function of human serum concentration. Solubility was determined in phosphate buffer 0.1 M, pH 7.4 ; anti-hCG serum 1 : 1,000 ; polyethylene glycol concentration 12.5% ; without adding gamma globulin (—) or with gamma globulin (···).

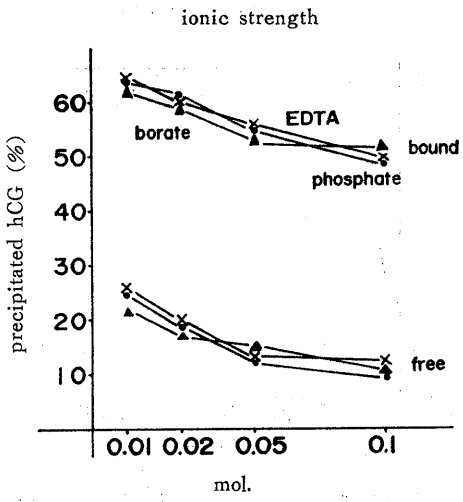

Fig. 4. Solubility of I^{125} labeled hCG as a effect of ionic strength. Solubility was determined in anti-hCG serum 1 : 30,000 ; polyethylene glycol concentration 12.5%; gamma globulin 1.0mg/ml.

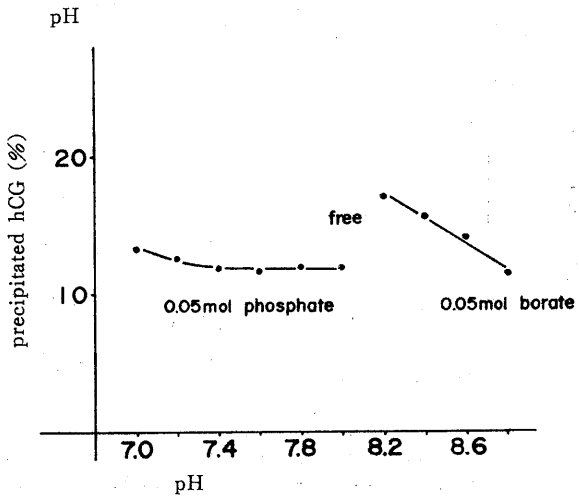

Fig. 5. Solubility of I^{125} labeled hCG as a effect of pH. Solubility was determined in anti-hCG 1 : 30,000 ; polyethylene glycol concentration 12.5% ; gamma globulin 1.0 mg/ml.

ated with the carrier protein. It was found that the optimal zone of polyethylene glycol to separate free from antibody bound hCG was 10–20%. The final concentration of the polymer was set at 12.5% for further investigation.

The solubility of the labeled hCG in aqueous polyethylene glycol was affected by the concentration of the proteins. The effects of the gamma globulin and of the human serum are illustrated in Fig. 2 and Fig. 3. The minimum concentrations required to complete precipitation of the antibody bound hCG was 0.5–1.0 mg/ml of gamma globulin from Green Cross, or 30–50 μl/ml of human serum. As the gamma globulin concentration was raised (especially beyond the level of 5.0 mg/ml), there was a decrease in the solubility of free hCG. By adding 0.1 ml of gamma globulin solution (1.0 mg/ml), the antibody bound hCG was precipitated at a constant level at any serum concentration. However, the precipitability of the free hCG was constant in the examined range of human serum, 10–200 μl/ml, whether gamma globulin was added or not.

The results of our experiments on the solubility in 0.01, 0.02, 0.05 and 0.1 molar of EDTA, phosphate and borate buffer are shown in Fig. 4. It was found that the precipitability of free and antibody bound hCG decreased with increasing in ionic strength of any buffer. The effect of pH on the solubility of free hCG is shown in Fig. 5. As the pH was lowered from 8.8 to 8.2 in the borate buffer, the solubility

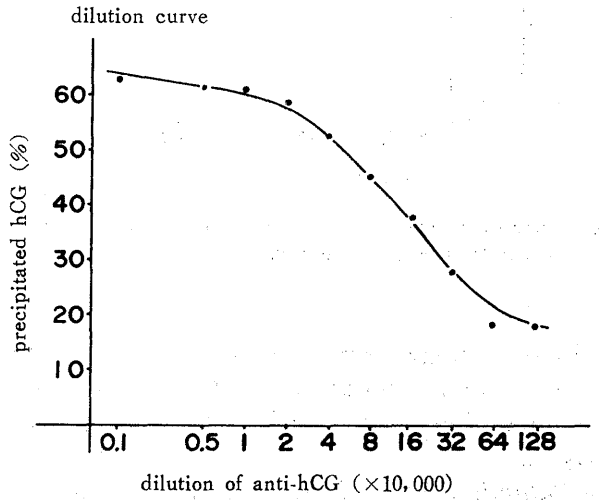

Fig. 6. Effect of dilution of the anti-hCG serum.

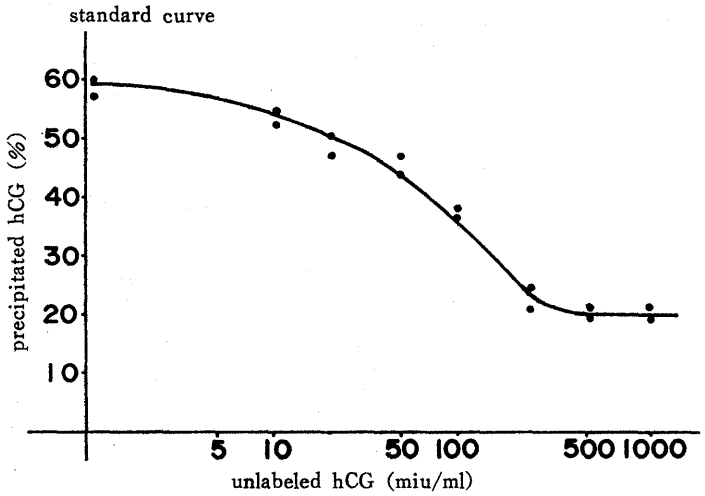

Fig. 7. A standard dose-response curve of hCG in polyethylene glycol method.

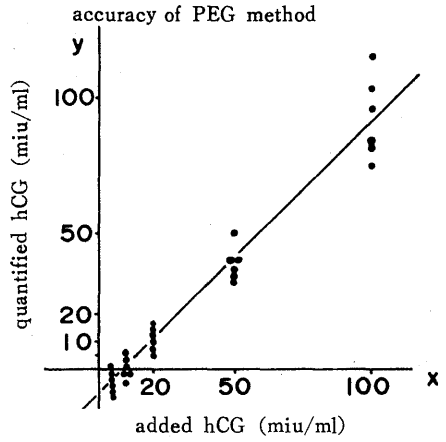

Fig. 8. Correlation between hCG added and hCG quantified in polyethylene glycol method.

decreased, but in the range from 7.4 to 8.0 in the phosphate buffer, little change was found. Phosphate buffer (0.05 mol, pH 7.4) was favorable to the separation of free from antibody bound hCG and was chosen for assay use.

Immunoprecipitability The effects of dilution of the anti-hCG guinea pig serum are presented in Fig. 6. The dilution required to bind 50 to 60% of ca. 10,000 cpm of I^{125}-hCG was found to be 1:30,000. The

standard curve of hCG is demonstrated in Fig. 7, which was prepared by plotting the percentage of radioactive hCG precipitated against the concentration of standard hCG. The linear portion of the assay curve was used for calculation. Various amounts of purified hCG ranging from 5 to 100 miu/ml were added to normal male serum for examination of accuracy of polyethylene glycol method. A linear relationship ($\gamma = 0.96$) was obtained between the added and quantified hCG, as shown in Fig. 8.

DISCUSSION

A fundamental requirment of any radioimmunoassay is a method for separation of free labeled antigen from that which is bound to specific antibodies. It has been proven that the double antibody method produces a clean separation and that this method is the one of the most successful separation techniques for radioimmunoassay. Daughaday and Jacobs[15] described the usefullness of the double antibody method and pointed out two disadvantages; skill and care were required in the aspiration of the supernatant solution, and the nature of the second antibody and the influence of human serum had to be investigated carefully.

On the other hand, methods based on chemical precipitation are inexpensive, simple, rapid and do not demand a labeled hormone of high specific activity. However, Arends[8] warned that special precautions had to be taken in chemical techniques to minimize the inevitable precipitation of free hormone and other proteins. In the chemical methods, the volume and concentration of the chemical substances producing precipitation and the carrier protein are very important[8,11,12].

According to our polyethylene glycol study, antibody bound hCG precipitated instantaneously and only in one incubation step. To obtain a good separation of antibody bound hCG, the following conditions are required; 1) a phosphate buffer, 0.05 mol pH 7.4, 2) a 12.5% final concentration of polyethylene glycol, 3) 1.0 mg/ml of gamma globulin as a carrier protein, and 4) 1.0 ml total volume of the incubation mixture before adding polyethylene glycol. The results which were obtained at 0.5 ml total volume were more variable than the results which were obtained at 1.0 ml total volume (the coefficient variations were 11.8%, 8.8%, respectively). The maximal amount of antibody bound hCG in the polyethylene glycol method is in agreement with the amount in the double antibody method[5]. The hCG radioimmunoassay using polyethylene glycol is unaffected by sodium chloride (not presented here) and protein concentration in physiological states.

Recently, the polyethylene glycol method has been described for precipitating antigen-antibody complexes in radioimmunoassays for cortisol[16,17], insulin[18,19], glucagon[20] and peptide hormones[12]. The polyethylene glycol method gives satisfactory results and is also adaptable for hCG radioimmunoassay.

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