[Chem. Pharm. Bull. 30(11)4114—4119(1982)]

Rapid Enzyme Immunoassay for Human Choriogonadotropin in Serum using Horseradish Peroxidase as a Label

KIYOSHI ZAITSU,[a] KENICHIRO NAKASHIMA,[b] SHUZO AKIYAMA[b] and YOSUKE OHKURA*,[a]

Faculty of Pharmaceutical Sciences, Kyushu University 62,[a] Maidashi, Higashi-ku, Fukuoka, 812, Japan and Faculty of Pharmaceutical Sciences, Nagasaki University,[b] Bunkyo-machi, Nagasaki, 852, Japan

(Received May 4, 1982)

A rapid sandwich-type enzyme immunoassay of human choriogonadotropin in 20 μl of serum is described. Anti-human choriogonadotropin β subunitantibody (IgG fraction) was coated on an acrylonitrile–butadiene–styrene copolymer bead. The bead was incubated with choriogonadotropin in serum at 30°C for 2 (or 3) h, and again incubated at 30°C for 2 (or 3) h with antibody-labeled horseradish peroxidase prepared by Nakane and Kawaoi's method, to form a sandwich-type immunocomplex. The enzyme activity of the complex on the bead was measured by the use of a sensitive fluorogenic substrate, 3-(*p*-hydroxyphenyl)propionic acid. The method permits one-day assay of 0.0125—64 ng (2.75—14080 mIU) of the hormone.

Keywords——enzyme immunoassay; human choriogonadotropin; anti-β subunit of human choriogonadotropin; horseradish peroxidase as label; sandwich method; 3-(*p*-hydroxyphenyl)propionic acid as fluorogenic substrate; rapid assay

Assay of human choriogonadotropin (HCG) in serum (or plasma) or urine is useful as a first-trimester placental function test and is important in the investigation of possible ectopic pregnancies.[1] Sensitive HCG assays can also be useful adjuncts to clinical evaluation in the diagnosis and follow-up study of patients with trophoblastic diseases such as hydatidiform moles or choriocarcinoma[2] and to research on some other HCG-secreting tumors of non-trophoblastic origin.

Enzyme immunoassay (EIA) of HCG was first reported by Weemen and Schuurs.[3,4] They examined three EIA systems, *i.e.*, double antibody solid phase, sandwich and immunoenzymometric methods, in which they used horseradish peroxidase (HRP) as a label enzyme with glutaraldehyde linkage. Of these methods, the double antibody solid phase method is most sensitive, but the sensitivity is not sufficient for the estimation of low levels of HCG in serum. Recently, a highly sensitive double antibody EIA method using β-galactosidase as a label was reported.[5] This method permits the assay of little as 4 μU of HCG (corresponding to 10 μl of 0.4 mIU/ml serum), but it requires a long time (about 5 d) to perform.

This paper describes a sandwich-type EIA method for serum HCG which can be performed within a day. The method uses an HRP-labeled antibody prepared by Nakane and Kawaoi's method,[6] and the HRP activity is measured by the use of a highly sensitive fluorogenic substrate for HRP, 3-(*p*-hydroxyphenyl)propionic acid (HPPA).[7]

Experimental

Materials and Apparatus——Water was deionized, then triply distilled and stored in a glass bottle protected from light. The second distillation was carried out from an alkaline $KMnO_4$ solution. H_2O_2 solution (Mitsubishi Gas Chemical, Japan) was standardized by acid permanganate titration. Tris(hydroxymethyl)aminomethane (Tris) (Ultrol grade) was purchased from Calbiochem (U.S.A.). HPPA was purchased from K & K Lab., Inc. (U.S.A.); it was dissolved in ether and passed through a column of neutral alumina (80 × 15 mm I.D.) to remove brownish impurities. The solvent was evaporated off, and the residue was repeatedly recrystallized from water to minimize background fluorescence. The intensities of the background fluorescence at an excitation wavelength of 320 nm and an emission wavelength of 404 nm were checked with

a solution of HPPA in 0.15 M Tris-HCl buffer (pH 8.5) after each recrystallization. HRP (300 purpurogallin units/mg) was purchased from Sigma Co. (U.S.A.). The activity of HRP was standardized by the fluorimetric method reported previously.[7] All other chemicals (unless otherwise noted) were of analytical grade. Purified HCG (4400 IU/mg, as compared with the second international standard of HCG)[8] and rabbit anti-HCGβ IgG were obtained from Mochida Pharm. Co. Ltd. (Japan). HCGβ was prepared by the method of Morgan and Canfield.[9] Anti-HCGβ serum was prepared by the method of Vaitukaitis *et al.*,[10] and the IgG fraction of the serum was prepared by three Na_2SO_4 fractionations (0.18, 0.12 and 0.12 saturation).

Fluorescence intensities were measured with a Hitachi MPF-4 spectrofluorimeter using quartz cells of 1×1-cm optical path length. The slit widths in the exciter and the analyzer in terms of wavelength were set at 2 and 10 nm, respectively.

Preparation of Anti-HCGβ IgG Coated Beads——Beads (gear-wheel-shaped acrylonitrile–butadiene–styrene copolymer; 10 mm in diameter, 6.65 mm in thickness, *ca.* 3.6 cm^2 in surface area; obtained from Mochida Pharm. Co. Ltd.) were coated with anti-HCGβ IgG as follows: a stock solution of anti-HCGβ (10 mg protein/ml of saline, 100 μl) was diluted with 0.05 M Na–K phosphate-buffered saline (pH 7.0) (PBS) to 200 ml. The antibody solution was added to 100 beads and incubated at 37°C for 2 d. The beads were washed with three 100-ml changes of saline and incubated at 37°C for 1 h with 100 ml of BSA (1 w/v %) dissolved in PBS (PBS–BSA)–NaN_3 (0.04 w/v %), then left standing at 4°C until use. Before use, the antibody-coated beads were washed with three 100-ml changes of saline. The beads were usable for at least 2 months when stored at 4°C.

Preparation of Anti-HCGβ IgG-HRP Conjugate——Anti-HCGβ IgG was chemically bound to HRP by Nakane and Kawaoi's method as follows: HRP (5 mg) was dissolved in 10 ml of freshly prepared 0.3 M Na_2CO_3 solution (pH 8.1) and mixed with 0.1 ml of ethanolic 1-fluoro-2,4-dinitrobenzene (1 w/v %) at 25°C. After 1 h, 1.0 ml of 0.06 M $NaIO_4$ solution was added with stirring and the mixture was left at 25°C for 30 min. Excess $NaIO_4$ was destroyed by the addition of 1.0 ml of aqueous ethyleneglycol (1 w/v %). The solution was left at 25°C for 1 h and dialyzed against three 1-liter changes of 0.01 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.5) at 4°C for 1 d. To the resulting solution, 0.5 ml of anti-HCGβ IgG (10 mg protein/ml of saline) was added with stirring at 25°C and the whole was left at 25°C for 3 h. Then, 5 mg of $NaBH_4$ was added and the mixture was kept at 4°C for 3 h. After dialysis against three 1-liter changes of PBS at 4°C for 1 d, the conjugate was purified by chromatography on a Sephadex G-200 column (75×1.37 cm I.D.) equilibrated with PBS using PBS as a mobile phase. The absorbances at 280 and 403 nm and the HRP activity of each fraction were measured by the methods reported previously.[7] Its immunoreactivity was also assayed by the present EIA procedure described below. Fractions No. 25 and 26 (each fraction was 1.6 ml, as shown in Fig. 1) were collected and stored at −20°C. When required for use, this solution was diluted 200 times with 0.05 M phosphate-buffered saline (pH 7.0) containing 1 w/v % BSA (PBS–BSA).

Procedure——HCG standard solutions were prepared in a concentration range of 0.625—3200 ng/ml (2.75—14080 mIU/ml) as PBS–BSA solution. All standards and samples were assayed in duplicate. Twenty μl of sample serum or a standard solution of HCG was added to 500 μl of PBS–BSA in a test tube (105×12.5 mm I.D.) containing one anti-HCGβ IgG coated bead and incubated at 30°C for 2 h with gentle mixing every 20 min (first reaction).

The incubated mixture was aspirated off, and the bead was washed twice with 4 ml of saline containing 0.005 w/v % Tween 20. Then 500 μl of PBS–BSA and 100 μl of anti-HCGβ IgG–HRP conjugate solution were added to the bead, which was again incubated at 30°C for 2 h with gentle mixing every 30 min (second reaction). The mixture was aspirated off, and the bead was washed three times with 4 ml of saline–Tween 20 solution, then subjected to measurement of HRP activity.

The washed bead was added to a mixture of 2.0 ml of 0.15 M Tris-HCl buffer (pH 8.5) and 1.0 ml) of 0.06 M aqueous HPPA solution, and then enzyme reaction was initiated by the addition of 100 μl of 0.04 M H_2O_2 with vigorous mixing. The mixture was incubated at 30°C for exactly 20 min. For the assay of HCG of 0.0125—2 ng/20 μl serum (2.75—440 mIU/ml), the first and second reactions were each carried out for 3 h, and the enzyme reaction for 40 min. The reaction was stopped by the addition of 100 μl of 5 w/v % aqueous Na_2SO_3. The fluorescence intensity was measured at an excitation maximum wavelength of 320 nm and an emission maximum wavelength of 404 nm.

Results and Discussion

In the preparation of anti-HCGβ antibody-coated beads, the adsorption of the antibody on the beads was interfered with by BSA if present in the antibody PBS solution. However, unfavorable non-specific binding of anti-HCGβ–HRP conjugate to the antibody-coated bead was inhibited by BSA (0.5—2 w/v %). Temperature affected the adsorption of antibody on the beads. At 37°C, the adsorption was almost complete in 2 d. The amount of adsorbed antibody decreased at temperatures lower than 30°C. Approximately maximal fluorescence

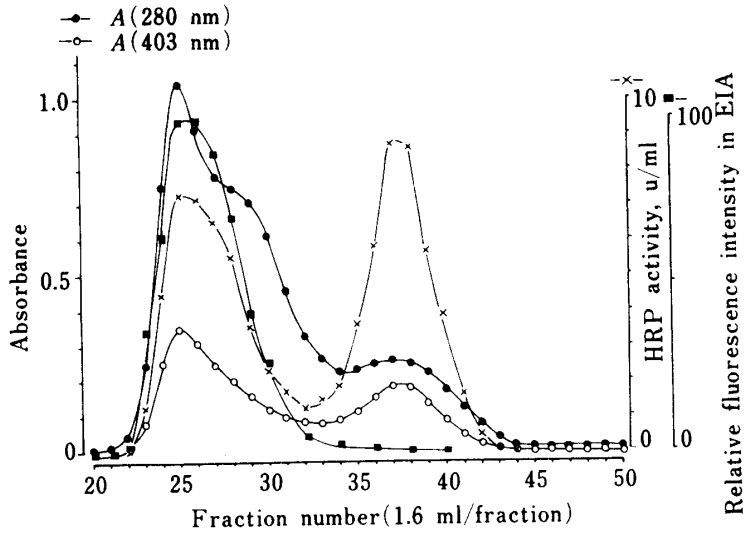

Fig. 1. Elution Profile of Anti-HCGβ–HRP Conjugate from a Sephadex G-200 (40—120 μm) Column

The column (71.5×1.37 cm I.D.) was equilibrated with 0.05 M Na–K phosphate-buffered saline (pH 7.0) at 4°C, and elution was carried out with the same buffer at 4°C at a flow rate of 4.5 ml/h.

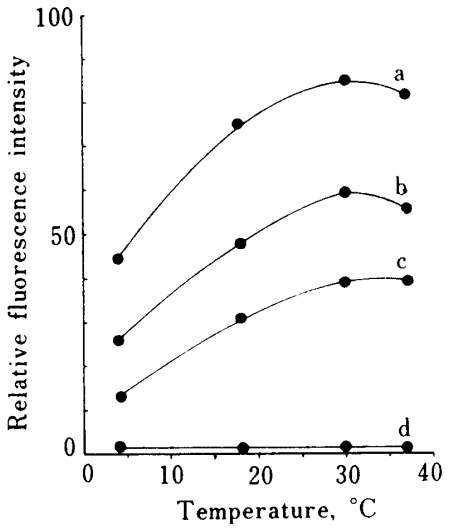

Fig. 2. Effect of Temperature in the Immuno-reactions (First and Second Reactions) on the Final Fluorescence Intensity

The amount of HCG per tube: a, 64 ng; b, 32 ng; c, 16 ng; d, 0.

intensities in the final assay mixtures were observed when the bead was prepared with 5—7.5 μg/ml antibody solution in a pH range of 6.0—8.0 in PBS.

Weemen and Schuurs[4] prepared anti-HCG–HRP conjugate (not anti-HCGβ–HRP conjugate) through a linkage with glutaraldehyde, and separated the conjugate from unreacted HRP and anti-HCG antibody by sucrose density gradient ultracentrifugation.

We obtained anti-HCGβ–HRP conjugate according to the method of Nakane and Kawaoi and successfully separated the resulting conjugate from HRP by gel chromatography, as shown in Fig. 1.

The conditions for the first and second reactions were investigated. As shown in Fig. 2, the highest fluorescence intensity was obtained with incubation at 30°C in both the first and second reactions.

The effect of pH of the media for the first and second reactions on the fluorescence intensity in the final assay mixture was examined in the range of 6.0—8.5. At a lower pH, the fluorescence intensity was slightly higher than that at higher pH, but the background fluorescence was also slightly higher. Thus pH 7.0 was selected as the optimum. As shown in Fig. 3, (A) and (B), incubation at 30°C for 2 or 3 h in both the first and second reactions provided a maximum fluorescence. The background fluorescence increased when the period of the second reaction was prolonged. The increase of the background fluorescence might be caused by an increment of non-specific direct binding of the antibody–HRP conjugate to the beads. Therefore 2-h incubation in the first and in the second reactions was used in the standard

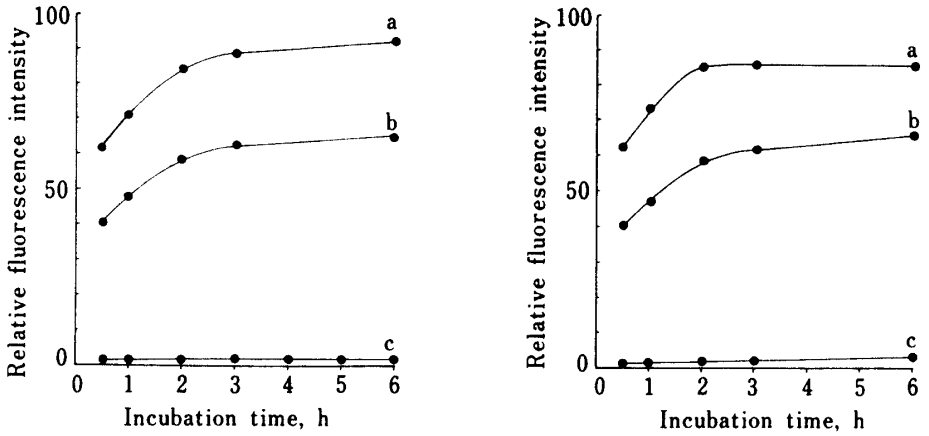

Fig. 3. Effect of Incubation Time in the Immuno-reactions (First and Second Reactions, A and B, respectively) on the Final Fluorescence Intensity

The amount of HCG per tube: a, 64 ng; b, 32 ng; c, 0.

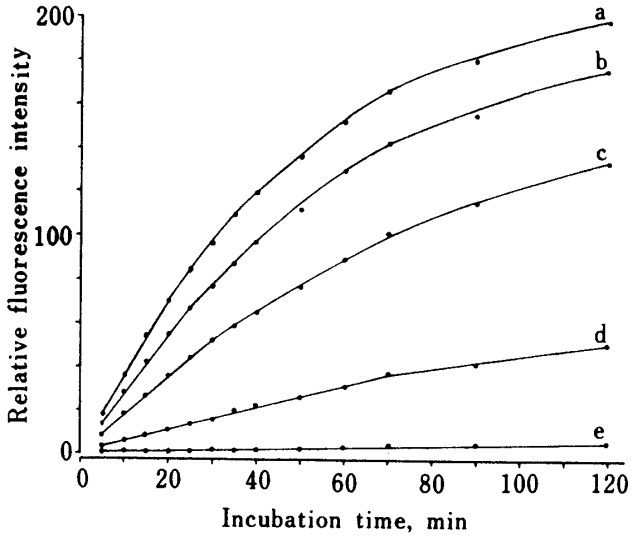

Fig. 4. Effect of Incubation Time in the Enzyme Reaction on the Fluorescence Development

The amount of HCG per tube: a, 64 ng; b, 32 ng; c, 16 ng; d, 4 ng; e, 0.

procedure. When a small amount (0.0125—2 ng) of HCG was to be assayed, however, 3-h incubation yielded a higher fluorescence intensity.

The enzyme reaction conditions were the same as described previously[11] except for incubation time. In the present method, incubation was carried out for 20 min (or 40 min for an assay of 0.0125—2 ng of HCG) at 30°C (Fig. 4).

Fig. 5, (A) and (B), show typical standard curves for 0.25—64 ng and 0.0125—2 ng of HCG, respectively. A linear relation was obtained from 0 to 1 ng (4.4 mIU). Weemen and Schuurs' procedure can only assay more than 60 mIU of HCG. The other EIA method reported[5] is about ten times more sensitive than the present method, but uses anti-HCG antibody (not anti-HCGβ antibody) and requires a long time. Thus, our method is relatively sensitive and rapid.

Parallel tests with a double-antibody radioimmunoassay (RIA) (HCG EIA Kit, HCG·I-125; Midorijuji Co., Japan) and the present method were performed on 30 different sera with

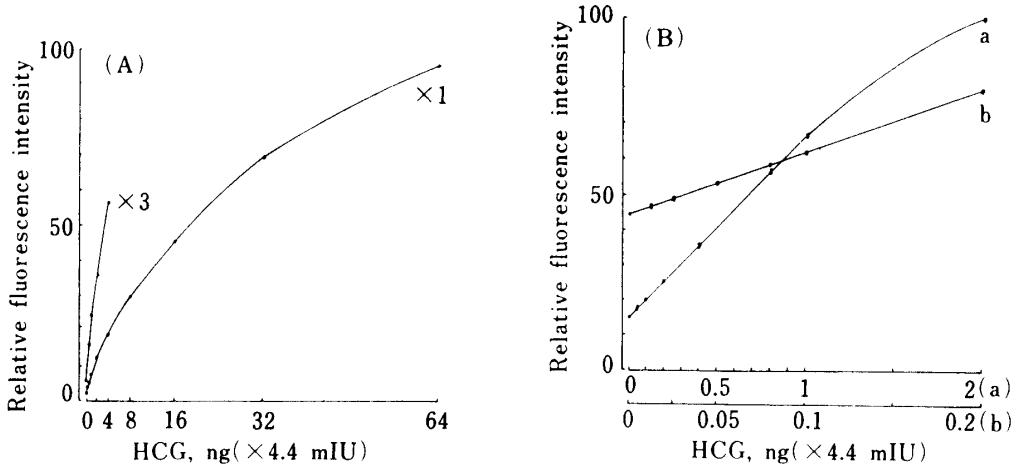

Fig. 5. Standard Curves for Serum HCG

×1 and ×3 indicate the sensitivity of the fluorimeter.

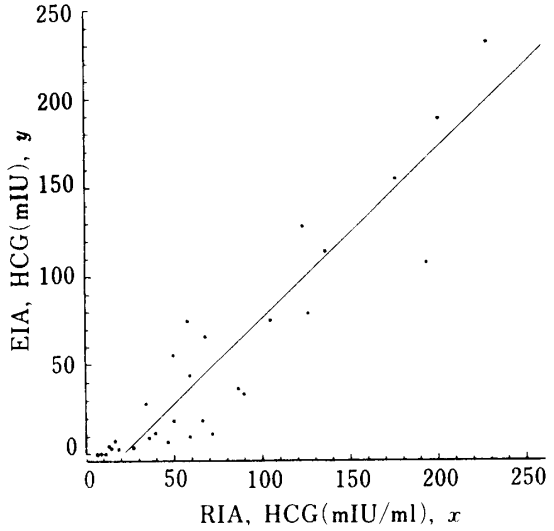

Fig. 6. Correlation between the Values of Serum HCG obtained by EIA (Present Method; amount of serum, 20 μl) and RIA (Midorijuji Co., HGG-I-125; amount of serum, 100 μl)

relatively low levels of HCG. The linear regression equation and correlation coefficient for the two methods were y(EIA)=0.97 x(RIA)−20.7 and 0.93, respectively (Fig. 6). The precision of the present method was studied with respect to reproducibility on 4 sera with 189, 107, 44 and 4.2 mIU/ml serum. The coefficients of variation were 4.3, 4.2, 3.8 and 3.5%, respectively (n=10). Standard solution containing 64, 32 and 16 ng of HCG in 20 μl of PBS–BSA (corresponding to 14080, 7040 and 3520 mIU/ml serum) were also examined (n=10). The coefficients of variation were 3.3, 2.8 and 3.0%, respectively.

Finally, we should like to stress that an antiserum generated against the hormone-specific β subunit of HCG is used as IgG fraction in the present procedure. This reduces the cross-reaction between HCG and other glycoprotein hormones, such as human luteinizing hormone, present in serum.[12]

Our method has several advantages over other methods, including conventional RIA. The reagents are easily prepared and are not subject to radioactive decay. Because the reactions are carried out with a large antibody-coated bead which is easily handled by means of a pincette, centrifugation is not necessary. The entire procedure can be carried out within a day. Therefore this method might be suited for clinical use.

Acknowledgement We thank Mrs. Yasuko Tsuruta (née Takanabe) for her skillful technical assistance. This work was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (56771062). Thanks are also due to Mochida Pharm. Co., Ltd. for the generous gift of some reagents.

References

1) C.D. Braunstein, W.G. Karow, W C. Gentry, J. Rason and M.E Wade, *Am. J. Obstet. Gynecol.*, **131**, 25 (1978).

2) E.S. Teoh, N.P. Das, M.Y. Dawood and S.S. Ratnam, *Acta Endocrinologica*, **70**, 791 (1972).
3) B.K. van Weemen and A.H.W.M. Schuurs, *FEBS Lett.*, **15**, 232 (1971).
4) B.K. van Weemen and A.H.W.M. Schuurs, *FEBS Lett.*, **43**, 215 (1974).
5) M. Kikutani, M. Ishiguro, T. Kitagawa, S. Imamura and S. Miura, *J. Clin. Endocrinol. Metab.*, **47**, 980 (1978).
6) P.K. Nakane and A. Kawaoi, *J. Histochem. Cytochem.*, **22**, 1084 (1974).
7) K. Zaitsu and Y. Ohkura, *Anal. Biochem.*, **109**, 109 (1980).
8) D.R. Bangham and B. Grab, *Bull. W.H.O.*, **31**, 111 (1964).
9) F.J. Morgan and R.E. Canfield, *Endocrinology*, **88**, 1045 (1971).
10) J. Vaitukaitis, J.B. Robbins, E. Nieschlag and G.T. Ross, *J. Clin. Endocr.*, **33**, 988 (1971).
11) K. Zaitsu, S. Eto and Y. Ohkura, *J. Clin. Lab. Inst. Reag. (Japan)*, **3**, 213 (1980).
12) H.C. Chen, G.D. Hodgen, S. Matsuura, L.J. Lin, E. Gross, L.E. Reichert, Jr., S. Birken, R.E. Canfield and G.T. Ross, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 2885 (1976).