Communications to the Editor

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CHEMILUMINESCENCE ENZYME IMMUNOASSAY OF 17α-HYDROXYPROGESTERONE USING GLUCOSE OXIDASE AND BIS(2,4,6-TRICHLOROPHENYL) OXALATE - FLUORESCENT DYE SYSTEM

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A highly sensitive enzyme immunoassay of 17α-hydroxyprogesterone has been developed. Glucose oxidase was used as a label enzyme and the double antibody solid phase method was adopted to separate the bound and free fractions after the immune reaction. In the assay of enzyme activity, hydrogen peroxide generated from glucose added as substrate was measured by the chemiluminescence reaction using bis(2,4,6-trichlorophenyl) oxalate and 8-anilinonaphthalene-1-sulfonic acid. The detection limit of this method is 0.5 pg/tube. The method is applicable to mass screening for congenital adrenal hyperplasia in neonates.

KEYWORDS———— chemiluminescence enzyme immunoassay; enzyme immunoassay; 17α-hydroxyprogesterone; bis(2,4,6-trichlorophenyl) oxalate; congenital adrenal hyperplasia; glucose oxidase

In the spite of the analytical advantages of radioimmunoassay (RIA), such as sensitivity and specificity, it has certain undesirable aspects including limited shelf life, potential health hazards, and the cost and inconvenience of radioactive waste disposal and licensing. As a result, there has been in recent years an increasing interest in alternative non-isotopic immunoassays. Particularly, enzyme immunoassay (EIA) using appropriate enzymes as labels instead of radioisotopes has become a clinically useful technique for the measurement of physiological concentrations of many clinically important compounds, hormones and drugs.[1] To increase the sensitivity of EIA, fluorophotometric and bio- or chemiluminescence methods are used.

We have reported the chemiluminescence EIAs of cortisol[2] and dehydroepiandrosterone[3] using peroxidase as the label and luminol-H_2O_2 as the substrate. In this communication we describe a new chemiluminescence EIA for 17α-hydroxyprogesterone (17-OHP). Rauhut[4] reported the chemiluminescence reaction of bis(2,4,6-trichlorophenyl) oxalte (TCPO) with H_2O_2 in the presence of a fluorescent dye. This system has been used for the determination of H_2O_2,[5,6] glucose,[5] uric acid[7] or lactate dehydrogenase.[8] This system was also applied to the detection of fluorophores, such as dansyl amino acids and catecholamines labeled with fluorescamine, on TLC[9] and HPLC.[10,11] To develope the highly sensitive EIA for 17-OHP based on this system, glucose oxidase (GOD) was used as the label and the double antibody solid phase method (DASP) was chosen for the separation of bound and free fractions after the immune reaction step. The 17-OHP-GOD conjugate was prepared by the mixed anhydride method from 17-OHP 3-(O-carboxymethyl)oxime and GOD as previously reported.[3] The proposed assay procedure is as follows:

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All solutions are diluted with 0.05 M phosphate buffer containing 0.1% BSA (PB-BSA). To each assay tube diluted anti-17-OHP antiserum (1 : 2.5 x 10^5) (0.1 ml), 17-OHP standard solution (0.1 ml) containing 0 1000 pg/ml, 0.05 M PB-BSA (0.1 ml) or one disc (3 mm I.D.) of dried blood sample spotted on filter paper, and one DASP bead are serially added and incubated overnight at 4 C. After incubation, 0.1 ml of 17-OHP-GOD conjugate solution (1 : 4 x 10^4) is added to each tube and incubated for 2 h at room temperature. The supernatant is aspirated off and the DASP-bead is washed twice with 0.1% Tween 20 - saline solution (2 ml) and saline solution (1 ml), and then incubated with 0.5 M glucose in 0.01 M acetate buffer (pH 5.0) (0.3 ml) overnight at 4 C. After incubation, 0.1 ml of the reaction solution is transferred to another glass tube, mixed with 0.02% 8-anilinonaphthalene-1-sulfonic acid (ANS) - 0.2 M barbital buffer solution (pH 9.0) (0.1 ml) and 5 mM TCPO - ethyl acetate solution (0.2 ml), and then the chemiluminescence intensity is measured with an Aloka Luminescence Reader (waiting time: 15 s, integrating time: 6 s).

Various factors affecting on the sensitivity of this EIA were examined. The fluorescent dyes tested in this study are ANS, fluorescin, fluorescein, 5-(2-aminoethylamino)-1-naphthalenesulfonic acid, 8-(2-aminoethylamino)-1-naphthalenesulfonic acid, and 9-anthraldehyde. The 5 mM TCPO - ethyl acetate solution (0.2 ml), 1 x 10^{-5} M H_2O_2 solution (0.2 ml) and 3 x 10^{-4} M fluorescent dye solution (pH 8.0) (0.1 ml) were mixed. The intensity of the chemiluminescence signal varied considerably with the fluorophores and solvents employed in the reaction. The S/N ratio of ANS, among the various fluorescent dyes tested, showed the highest value. Therefore, ANS was used as the fluorescent dye for the assay system. The optimal concentration of ANS, the pH and the other conditions, such as the dilution of antiserum and 17-OHP-GOD conjugate solution, were selected as described above.

Typical standard curves are shown in Fig. 1. The solid line is the curve obtained when the standard 17-OHP solutions are used. The coefficients of variation at each level of 17-OHP (n = 5) ranged from 1.7 to 8.9%. The detection limit of this EIA was 0.5 pg/assay tube, which corresponds to 5 pg/ml (16 fmol/ml). This is slightly lower than the limits found for RIA, such as 5 pg/tube,[12] 10 pg/tube,[13] and 2 pg/tube,[14] The results of whithin-assay and between-assay using blood samples are shown in Table 1. The recovery from plasma samples prepared by adding 5 or 10 ng of 17-OHP per ml was determined using the dichloromethane extraction method; the values (n = 5) were 104.4% (CV = 8.7%) and 100.7% (CV = 6.8%), respectively. Therefore, there was no evidence that the dichloromethane extraction method interfered in the assay.

The dotted curve in Fig. 1 was constructed in the presence of one disc of dried blood sample spotted on filter paper. The curve is higher compared with the curve of the buffer system. Therefore, the standard curve should be constructed by adding one disc of steroid free dried blood on filter paper to the standard solution for the assay of 17-OHP in dried blood samples.

The most important application of EIAs is the mass screening test in which a large number of samples must be assayed within the shortest possible time. Though

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the chemiluminescence EIA using peroxidase-luminol-H_2O_2 is highly sensitive, serious interference is found in assay of dried blood samples. Therfore, this method can not be applied to the direct assay of such samples. In contrast, the chemiluminescence EIA based on the glucose oxidase-glucose-TCPO-ANS system can be used for such an assay. 17-OHP, an adrenal steroid, is increased in the serum of patients with congenital adrenal hyperplasia (CAH), a disease secondary to an enzymatic block in the synthesis of cortisol. The measurement of 17-OHP in newborn infants is used as the initial diagnosis of CAH.[12-14] Therefore, the present EIA method was applied to the mass screening test for CAH. In a preliminary screening test, 40 samples were assayed by the present method and the fluorescence EIA method[15] which correlated well with the RIA method reported by Kambegawa et al.[14] The correlation coefficient was 0.931 and the regression line was Y (the present method) = 1.25 X (the fluorescence method) - 1.23.

The EIA method for the determination of 17-OHP based on the chemiluminescence reaction of TCPO-H_2O_2-ANS is simple and sensitive, and generally applicable to EIAs for the determination of various hormones and drugs in complex biological fluids.

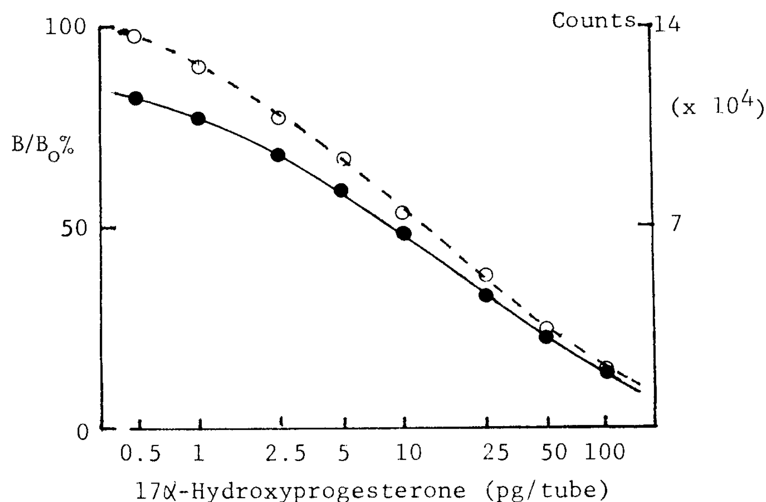

Fig. 1 Standard Curves of 17α-Hydroxyprogesterone

—●— Buffer system - -○- - Dried blood disc system

Table 1. Precision of Chemiluminescence EIA for 17α-Hydroxyprogesterone

Sample	Within-assay (n = 5)		Between-assay (n = 5)	
	A	B	C	D
Mean value (ng/ml)	8.9	19.9	10.8	20.7
S.E. (ng/ml)	0.9	2.1	2.0	3.5
C.V. (%)	10.3	10.6	18.5	16.9

S.E. : standard error of mean.

C.V. : coefficient of variation.

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