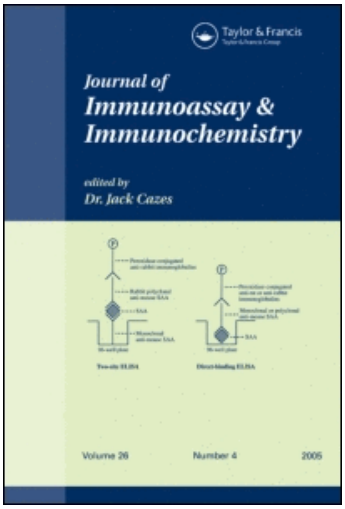

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FLUORESCENCE ENZYME IMMUNOASSAY OF 21-DEOXYCORTISOL IN PLASMA AND DRIED BLOOD SAMPLE ON FILTER PAPER

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ABSTRACT

An enzyme immunoassay of 21-deoxycortisol (21-DOF) in plasma and dried blood spotted on filter paper has been developed. 21-DOF was conjugated to horseradish peroxidase by the mixed anhydride method. Separation of free and bound fractions was done by the use of insolubilized antibody, prepared by coating polyacetal beads with purified IgG of goat anti-rabbit IgG serum. The enzyme activity was measured by the fluorophotometric method using 3-(p-hydroxyphenyl)propionic acid and H_2O_2 as substrates. The sensitivity of the present method was 0.5 pg/tube for 21-DOF. The intra- and interassay coefficients of variation were 3.2 and 8.2, and 7.9 and 9.2% respectively. The present enzyme immunoassay could be applied to mass-screening for congenital adrenal hyperplasia.

INTRODUCTION

It has been reported that the disorder of congenital adrenal hyperplasia (CAH) is caused by a hereditary 21-hydroxylation defect in a very high percentage of cases. In newborns, measurement of 17α-hydroxyprogesterone (17-OHP) using radioimmunoassay (RIA)

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is used in the initial diagnosis of CAH (1). Fukushima et al. (2) developed also RIA for 21-deoxycortisol (21-DOF) in plasma and reported that measurement of 21-DOF is useful for the diagnosis of CAH as well as 17-OHP. However, the reported RIA method is less sensitive and is not applicable to the determination of 21-DOF in one dried blood disc (3 mm in diameter); equivalent to about 3.6 μl of whole blood. Furthermore, RIA method is not suitable for large scale mass-screening test for CAH of newborn babies, because RIA has the disadvantages of requiring disposal of radioactive materials. We have developed a fluorescence enzyme immunoassay of 17-OHP using peroxidase as the enzyme label and applied it to the mass-screening for CAH of newborn babies (3). In this paper, we have attempted to develop a highly sensitive EIA for the assay of 21-DOF in dried blood samples in order to establish the mass-screening for CAH based on the more definitive diagnosis with regard to steroid 21-hydroxylase deficiency with and without 11β-hydroxylase deficiency.

MATERIALS AND METHODS

Materials

21-DOF was purchased from IKAPHARM RAMAT-GAN, (Israel), 3-(p-hydroxyphenyl)propionic acid (HPPA) was from K & K Laboratories (U.S.A.), 30% hydrogen peroxide and other chemicals were from Tokyo Chemical Industry Co. (Tokyo, Japan). Horseradish peroxidase (grade II, 250 IU/mg) was obtained from Toyobo Co. (Osaka, Japan). 21-DOF-3-carboxymethyl oxime (21-DOF-3-CMO), two

anti-21-DOF anti-sera, prepared by raising against 21-DOF-3-CMO-BSA conjugate and 21-DOF-6 -hemisuccinate-BSA conjugate, and 1,2-[^{3}H]-21-DOF (50 Ci/mmol) were kindly supplied by Teikoku Hormone Co. (Kawasaki, Japan). Goat anti-rabbit IgG antiserum and polyacetal beads used as the solid phase were also provided from Fuji Revio Co.. All solvents and chemicals were of reagent grade.

Other steroids were purchased from Sigma Chemical Co. (U.S.A.) and sulfates of several steroids were kindly supplied by Prof. T. Numbara (Tohoku University) and Prof. Kirk (University of London).

Buffer solutions

A 0.05 M phosphate buffer containing 0.9% sodium chloride (pH 7.0) (PBS), a 0.05 M phosphate buffer containing 0.9% sodium chloride and 0.1% BSA (pH 7.0) (PBS-BSA), and 0.05 M phosphate buffer (pH 7.4) were used in this study.

21-DOF Standard solution

A stock solution of 21-DOF in ethanol (100 ug/ml) was serially diluted with PBS-BSA before use.

21-DOF-HRP conjugate

21-DOF-HRP conjugate was prepared by the mixed anhydride method from 21-DOF-3-CMO and HRP according to the previous (3). 21-DOF-3CMO (3 mg) was dissolved in dioxane (0.2 ml), then tri-n-butylamine (10 μl) and isobutylchlorocarbonate (4 μl) was added. After incubation for 30 minutes at 10°C, the reaction solution was mixed with the cooled HRP solution (10 mg/ml). The mixture was kept at pH 8.0$\sim$8.5 for 4 hours in an ice-water bath. The

resulting reaction mixture was dialyzed against 0.05 M PBS and then chromatographed on a Bio-Gel P-200 column (100 x 1 cm I.D.) using 0.05 M PBS as eluent. The absorbances at 280 nm and 480 nm, and the HRP activity of each fraction (1.0 ml) was measured by the previous method. The immunoreactivity of each fraction was assayed by the present EIA method described below. Fractions from 39 to 43 were diluted five-fold with 0.05 M PBS-BSA and stored at 4°C until used.

Double antibody solid phase (DASP) beads

DASP beads were prepared as reported in the previous paper (3). Goat anti-rabbit IgG antiserum was purified by affinity chromatography on a column packed with rabbit IgG-Sepharose 4B, then diluted with phosphate buffer (50 mM, pH 7.4) to 22 μg/ml. After polyacetal beads (cogwheel type, 6 mm in diameter and 6 mm thick) were immersed overnight in the diluted goat anti-rabbit IgG solution at room temperature, the anti-rabbit IgG coated beads were coated with 3 g/l BSA-saline solution for 1 h at room temperature and stored at 4°C until used. Just before use, the DASP beads were washed with 0.05 M PBS-BSA.

EIA for the determination of 21-DOF in plasma and dried blood samples

All solutions were diluted with 0.05 M PBS-BSA. All standards and samples were assayed in duplicate. Discs, 3 mm in diameter, were punched out from dried blood samples mailed to screening center (National Center for Nervous, Mental and Musclar Disorders, Kodaira, Tokyo, Japan).

Extraction of 21-DOF from plasma sample

A plasma sample (20 ul) was diluted by adding water (0.5 ml), extracted with CH_2Cl_2 (5 ml) using a **Vortex** type mixer and the water layer was aspirated off. The CH_2Cl_2 extract (4 ml) was then transferred into another test tube and evaporated to dryness under nitrogen gas stream. The residue was dissolved by adding 0.05 M PBS-BSA (0.5 ml) and 0.1 ml of the resultant solution was subjected to the following EIA.

Extraction of 21-DOF from dried blood sample

Two discs of dried blood sample were transferred into a test tube (50 mm x 6 mm, I.D.) containing water (0.5 ml) and incubated overnight at room temperature. 21-DOF eluted from discs was extracted with ether (5 ml) by mixing for 2 min. The water layer was discarded and the ether layer was washed with water (0.5 ml). The ether layer (4 ml) was then transferred into another test tube and evaporated to dryness under nitrogen gas stream. The residue was dissolved by adding 0.05 M PBS-BSA (0.32 ml) and each 0.1 ml of the resultant solution was then subjected to the following EIA and RIA.

EIA procedure of 21-DOF

The assay procedure was performed in duplicate in disposable polystyrene test tubes (50 mm x 6 mm, I.D.) by sequential addition of 0.1 ml of anti-21-DOF antiserum (1 : 5000), 0.1 ml of 21-DOF standard solution or sample solution prepared from plasma or dried blood sample, 0.1 ml of 21-DOF-HRP conjugate solution (1 : 1000), 0.1 ml of 0.05 M PBS-BSA, and one DASP bead. The assay mixture

was agitated by a Vortex type mixer and incubated overnight at 4°C. After incubation, the reaction solution was aspirated off and then the bead was washed three times with each 2 ml of saline. To the washed bead 500 µl of PBS, 50 ul of 0.01% H_2O_2 solution and 50 ul of 0.5% HPPA solution were added serially and mixed well. After incubation for 1 hr at room temperature, the reaction was stopped by addition of each 50 ul of 1.25% KCN solution and 1 N NaOH solution, or by addition of 100 µl of 3% NaN_3 solution. The fluorescence intensity was measured at excitation wavelength of 320 nm and emission wavelength of 405 nm using an automatic fluoro-photometer for screening test (Auto FP-1, Fuji Revio Co., Tokyo, Japan).

RIA of 21-DOF

The RIA method used here was as described by Fukushima et al. (2), using 1,2-[^{3}H]21-DOF as the labeled compound and the anti-21-DOF antiserum against 21-DOF-6-hemisuccinate-BSA, without the clean-up step of chromatography.

RESULTS

Optimisation studies

To establish the optimal standard method of EIA for 21-DOF, we examined what dilutions of anti-21-DOF antiserum and 21-DOF-HRP conjugate yielded optimal fluorescence intensity. In general, the fluorescence intensity at B_o decreases with increasing dilution of antiserum, the sensitivity of the assay increases, however, increasing dilution also decreases precision because of a decrease

in the amount of enzyme labeled conjugate bound to the DASP bead. B/B_o values at the level of 10 pg of 21-DOF in the assay using various dilutions of anti-21-DOF antiserum and 21-DOF-HRP conjugate are illustrated in Table 1. As shown in Table 1, when used 1000 2000 fold dilution of 21-DOF-HRP conjugate and 5000-fold dilution of anti-21-DOF antiserum, B/B_o % at 10 pf pg of 21-DOF decreased to 30—35% from 100%. Therefore, balancing between sensitivity and precision, we chose to use 1000-fold dilution of 21-DOF-HRP conjugate and 5000-fold dilution of anti-21-DOF antiserum for this study.

Standard curves

The standard curves of 21-DOF obtained under the optimal conditions are shown in Fig. 1. The range of standard curve obtained with anti-21-DOF-3-CMO-BSA antiserum and anti-21-DOF-6α-hemisuccinoxy-BSA antiserum were from 5 pg to 500 pg/tube and from 0.5 pg to 100 pg /tube, respectively. The standard curve of RIA using 1,2-[^{3}H]-21-DOF as a label and anti-21-DOF-6α-hemisuccinoxy-BSA antiserum is also shown in Fig. 1. The assay range is from 2.5 pg to 50 pg/tube.

Specificity

The specificities of antisera used here were assessed with 21-DOF-3-CMO-HRP conjugate. Table 2 lists the cross-reactivities of these antisera with various steroids. The method using anti-21-DOF-6-α-hemisuccinate-BSA antiserum was more specific than the homologous EIA method.

TABLE 1

Comparison of the Binding of 21-DOF-HRP Conjugate to DASP Bead

Dilution of Antiserum *	Dilution of 21-DOF-HRP Conjugate		
	1 : 500	1 : 1000	1 : 2000
1 : 1000	82	68	63
1 : 2000	57	54	33
1 : 5000	50	35	30

Figures in table indicate the B/B_0 % at 10 pg of 21-DOF

* : Anti-21-DOF-6α-hemisuccinate-BSA

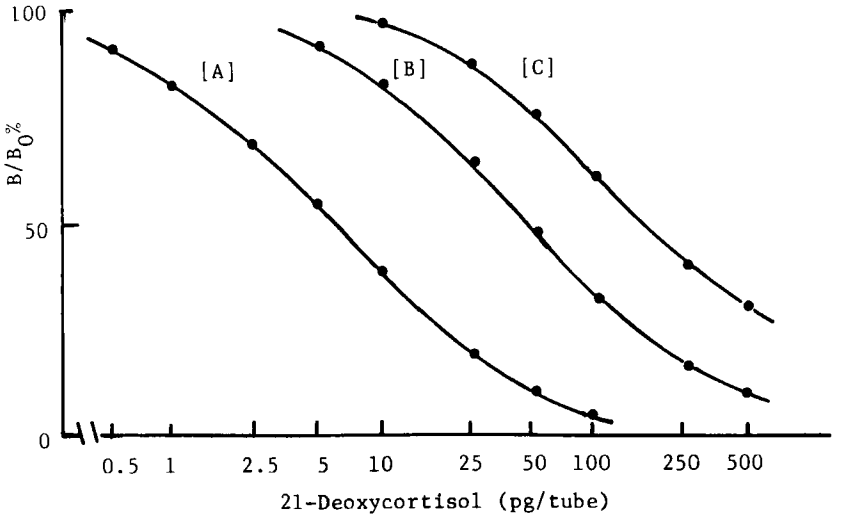

Fig. 1. Standard Curves of 21-Deoxycortisol by EIA and RIA

A : EIA using Anti-21-DOF-6α-hemisuccinate-BSA
B : EIA using Anti-21-DOF-3-CMO-BSA
C : RIA using Anti-21-DOF-6α-hemisuccinate-BSA

TABLE 2

Cross Reaction of Antisera to 21-Deoxycortisol

Steroids	Anti-21-DOF-3	Anti-21-DOF-6
21-Deoxycortisol	100.00 %	100.00 %
17α-Hydroxyprogesterone	131.40	19.00
Cortisol	5.47	1.05
Progesterone	4.00	0.01
11-Deoxycortisol	2.63	0.02
Prednisolone	2.19	1.40
17α-Hydroxypregnenolone	1.20	1.03
Corticosterone	0.23	0.57
11-Deoxycorticosterone	0.14	0.01
20α-Dihydroprogesterone	0.14	0.01
Pregnenolone	0.13	0.01
16α-Hydroxyprogesterone	0.10	0.01
Cortisone	0.06	0.10
Tetrahydrocortisol	0.01	0.01
Tetrahydrocortisone	0.01	0.01
17α-Hydroxypregnenolone-3-sulfate	-	1.71
Pregnenolone-3-sulfate	-	0.60
5β-Pregnene-3α,20α-diol-3-sulfate	-	0.01
5β-Pregnene-3α,20α-diol-20-sulfate	-	0.01
5β-Pregnene-3α,20α-diol-disulfate	-	0.01

Anti-21-DOF-3 : Antiserum against 21-DOF-3-(O-carboxymethyl)oxime-BSA

Anti-21-DOF-6 : Antiserum against 21-DOF-6α-hemisuccinate-BSA

- : No data

Recovery study

A known amount of 21-DOF was added to plasma samples containing endogeneous 21-DOF. As shown in Table 3, recoveries were 95% for 5 ng/ml and 105% for 10 ng/ml, respectively.

TABLE 3

Recovery of 21-Deoxycortisol from Plasma

Added (ng/ml)	Found (mg/ml)	CV (%)	Recovery (%)
-	1.43 ± 0.18	12.6	-
5.0	6.18 ± 0.47	7.6	95.0
10.0	11.95 ± 0.79	6.6	105.0

n = 6

Precision

Intra- and interassay precision (CV) were evaluated by testing five replicates each of low and high concentrations of plasma samples. Analysis of these samples on the same day gave mean values of 1.71 (SD ± 0.14) and 3.43 (SD ± 0.11) ng/ml, respectively. The mean values determined over a five-day period were 1.85 (SD ± 0.17) and 3.43 (SD ± 0.27) ng/ml, respectively. Thus, the intra-assay CVs were 8.2% and 3.2%, and interassay CVs were 9.2% and 7.9%, respectively.

Correlation with the RIA for 21-DOF

Using the present fluorescence EIA (X) and the RIA (Y), we assayed, in duplicate, 34 plasma samples. The linear correlation coefficient between the 21-DOF values determined by those two methods was 0.92 (slope = 0.925, y-intercept = 0.08).

Comparison between 21-DOF values in plasma and dried blood disc

The 21-DOF values in plasma and dried blood discs prepared from the same speciments were also compared to assess whether 21-DOF values in dried blood discs reflects to those in plasma samples. As illustrated in Fig. 2, the regression analysis

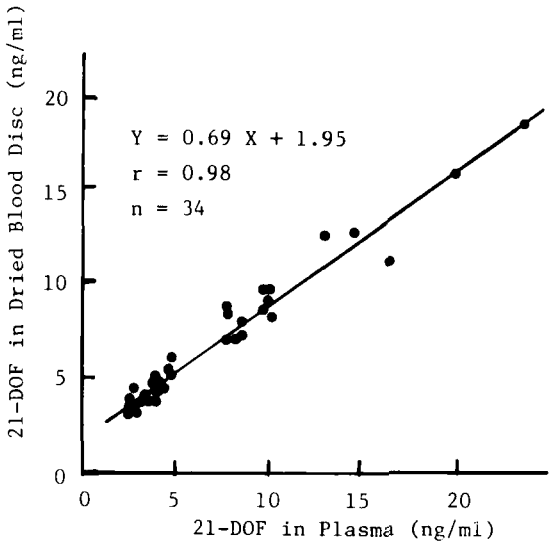

Fig. 2. Correlation between 21-DOF Values in Dried Blood samples and Plasma Samples

showed a good correlation between the values obtained with dried blood discs and plasma samples.

Preliminary application to mass-screenings

Dried blood samples from 100 neonates (ages five to seven days) were analyzed, in duplicate, by the present EIA method. As shown in Fig. 3, the histogram of 21-DOF values in the samples shows a normal distribution, with a mean of 21.2 ± 5.2 pg/disc.

DISCUSSION

The pilot screening programs for congenital hypothyroidism using EIA have been started in Japan. The fluorescence EIA of 17-OHP has

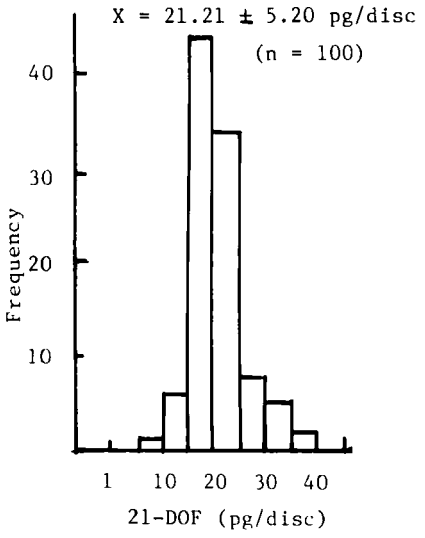

Fig. 3. Histogram of 21-DOF Values in Dried Blood Samples

also been developed in our laboratory (3) and been applied to the mass-screening test for congenital adrenal hyperplasia. However, for more definitive diagnosis of the enzyme defect, simultaneous measurement of 21-DOF in blood is necessary.

In this study, we have developed a highly sensitive EIA of 21-DOF using peroxidase as the label enzyme. Various analytical factors were examined to obtain the optimal conditions for the assay.

EIAs for haptens, such as steroids and drugs may be "homologous", in which the same derivatives of hapten are used both preparing the hapten derivative-carrier protein and the hapten enzyme conjugate, or "heterologous", in which different hapten derivatives (bridge or site) are used. Van Weemen and Schuurs (4) suggested that the sensitivity of EIA for estrogen could be considerably improved by

using heterologous systems. Hosoda et al. (5) examined many steroid derivatives and their antisera and reached the same conclusion. We have also obtained the similar results in the EIAs for cortisol and dehydroepiandrosterone (6,7). In this study, we used two different anti-21-DOF antisera, prepared by raising against 21-DOF-3-CMO-BSA and 21-DOF-6α-hemisuccinoxy-BSA respectively, and 21-DOF-HRP conjugate prepared from 21-DOF-3-CMO. As shown in Fig. 1, the heterologous assay system using anti-21-DOF-6α-hemisuccinate-BSA antiserum seemed to be more sensitive than the homologous system using anti-21-DOF-3-CMO-BSA antiserum.

As shown in Table 2, anti-21-DOF-6α-hemisuccinoxy-BSA antiserum was more specific than the homologous system. However, anti-21-DOF-6α-hemisuccinate-BSA antiserum showed significant cross reactions with 17-OHP (19.0%), cortisol (1.05%), prednisolone (1.40%), 17α-hydroxypregnenolone (1.03%) and 17α-hydroxypregnenolone-3-sulfate (1.71%). Therefore, in order to obtain the accurate values of 21-DOF, the chromatographic separation of 21-DOF from cross-reacting steroids in the sample using Celite column chromatographic technique reported by Fukushima et al. (2) would be required prior to immunoassay step in the assay.

The sensitivity of the present EIA method is higher than that of the RIA method, as shown in Fig. 1. Furthermore, the correlation between EIA and RIA is good; correlation coefficient $r = 0.93$. Dried blood and plasma samples from the same subjects were also assayed by the EIA. There was a high correlation ($r = 0.98$) between the results of both samples.

In conclusion, the present EIA method for 21-DOF is superior to the RIA because of its high sensitivity, good reproducibility, absence of disposal problems and its use of less expensive apparatus. The present method will be applicable to the purpose of mass-screening for congenital hyperplasia of neonates as well as the fluorescence EIA of 17-OHP.

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