Fluorescence Enzyme Immunoassay of 17α-Hydroxyprogesterone in Dried Blood Samples on Filter Paper and Its Application to Mass Screening for Congenital Adrenal Hyperplasia

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An enzyme immunoassay of 17α-hydroxyprogesterone (17-OHP) in dried blood spotted onto filter paper has been developed. 17-OHP was conjugated to horseradish peroxidase by the mixed anhydride method. Separation of free and bound 17-OHP-peroxidase conjugate was done by the use of insolubilized antibody, prepared by coating polyacetal beads with purified immunoglobulin G (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 1.0 pg/tube for 17-OHP. The intra- and interassay coefficients of variation were 5.4—7.0% and 8.3—14.7%, respectively. The present enzyme immunoassay method could be applied to mass screening for congenital adrenal hyperplasia.

Keywords——fluorescence enzyme immunoassay; enzyme immunoassay; 17α-hydroxyprogesterone; mass screening; congenital adrenal hyperplasia; peroxidase

In newborns, measurement of 17α-hydroxyprogesterone (17-OHP) is used in the initial diagnosis of congenital adrenal hyperplasia (CAH) due to steroid 21-hydroxylase deficiency.[1-3] To facilitate mass screening for CAH, Pang et al.[4] developed a radioimmunoassay (RIA) of 17-OHP which uses dried blood spotted on filter paper. Subsequently, several 17-OHP assays using the RIA method were reported.[5-7] The RIA method is sensitive, but most of these methods call for an extraction step with organic solvent and the extraction technique is not suitable for large-scale mass screening. Moreover, RIAs present problems related to the disposal and radiation hazard of isotopes. Thus, there is a need for non-isotopic methods. In 1972, Van Weemen and Schuurs[8] reported an enzyme immunoassay (EIA) of steroid using enzyme as a label instead of radioisotope. Since then, a number of studies have been done on EIA for various hormones and drugs;[9] in some instances, the sensitivity of EIA surpassed that of RIA. Kohen et al.[10] developed an EIA of 17-OHP. Their method does not require the separation of bound and free fractions, but it is less sensitive than the usual RIA and is not applicable to the determination of 17-OHP in dried blood samples. Therefore, we attempted to develop a highly sensitive EIA for the assay of 17-OHP in dried blood samples.

Materials and Methods

Materials——17-OHP and 17-OHP-3-O-carboxymethyl oxime (17-OHP-3-CMO) were purchased from Sigma Chemical Co. (St. Louis, Mo. U.S.A.), 3-(p-hydroxyphenyl) propionic acid (HPPA) was from K & K Laboratories (U.S.A.), and 30% hydrogen peroxide and other chemicals were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Horseradish peroxidase (grade II, 250 IU/mg) was obtained from Toyobo Co. (Osaka, Japan). Anti-17-OHP antiserum, which was raised against 17-OHP-3-CMO-BSA conjugate, was kindly supplied by Teikoku Hormone Co.

(Kawasaki, Japan), and goat antiserum against rabbit IgG by Fuji Hormone & Pharmaceutical Co. (Tokyo, Japan). The polyacetal beads used as the solid phase were also generously provided by Fuji Hormone & Pharmaceutical Co. (Tokyo, Japan). All solvents and chemicals were of analytical reagent grade.

Buffer Solution——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 0.55% sodium phosphate solution (pH 7.4) were used in this study.

17-OHP Standard Solution——A stock solution of 17-OHP (100 μg/ml) in ethanol was serially diluted with PBS–BSA.

17-OHP-HRP Conjugate——17-OHP-HRP conjugate was prepared from 17-OHP-3-CMO and HRP by the mixed anhydride method. 17-OHP-3-CMO (3 mg) was dissolved in dioxane (0.2 ml), then tri-n-butylamine (10 μl) and isobutylchlorocarbonate (4 μl) were added. The resultant solution was gently stirred for 30 min at 10 °C, and then mixed with cooled HRP solution (10 mg/ml). The mixture was kept at pH 8.0—8.5 for 4 hr 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 × 1 cm, i.d.) equilibrated with the same buffer. The absorbances at 280 and 430 nm and the HRP activity of each fraction (1.3 ml) were measured by the methods reported previously.[11] The immunoreactivity of each fraction was also assayed by the present EIA method described below. Fractions from No. 13 to 16 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 elsewhere.[11] Goat anti-rabbit immunoglobulin G (IgG) was purified by affinity chromatography and diluted with 0.55% sodium phosphate solution (pH 7.4.). Polyacetal beads were immersed overnight in the diluted goat anti-rabbit IgG solution (22 μg/ml) at room temperature, then the anti-rabbit IgG-coated beads were coated with 0.3% BSA–saline for 1 h at room temperature and stored in the same solution at 4 °C until used. The DASP beads were washed with 0.05 M PBS just before use.

EIA for the Determination of 17-OHP in Dried Blood Spotted on Filter Paper——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 filter papers carrying steroid-free dried blood or samples.[15]

One disc of dried blood sample was placed into each assay tube, which contained anti-17-OHP antiserum (1: 160000) (0.1 ml) and 0.05 M PBS–BSA (0.3 ml). For the standard curve, one disc of filter paper carrying steroid-free dried blood was placed into each assay tube, which contained anti-17-OHP antiserum (1: 160000) (0.1 ml), 17-OHP standard solution (0.1 ml, 0—100 pg), and 0.05 M PBS–BSA (0.2 ml). One DASP bead was added to each tube and the contents were mixed well. After overnight incubation at 4 °C, 17-OHP-HRP conjugate solution (1 : 20000) (0.1 ml) was added to each assay tube, followed by 2 h incubation at room temperature. The supernatant was aspirated off and the DASP-bead was washed with 0.1% Tween 20-saline solution (2 ml) and then with saline (2 ml). The washed DASP bead was subjected to assay of peroxidase activity, using the following fluorophotometric method.

Fluorophotometric Methods for the Assay of Peroxidase Activity——After 0.05 M PBS (500 μl), 0.01% H_2O_2 (50 μl) and 0.5% HPPA solution (50 μl) had been added serially to the assay tube containing the washed bead, the mixture was incubated for 1 h at room temperature. The enzyme reaction was stopped by adding 1.25% KCN solution (50 μl) and 0.1 M NaOH solution (50 μl). The fluorescence intensity was measured at excitation and emission wavelengths of 320 and 405 nm, respectively, using a Hitachi MPF 2A spectrofluorophotometer..

RIA for the Determination of 17-OHP——The RIA method used here was as described by Kambegawa *et al.*,[6] using [³H] 17-OHP as the labeled compound and the same antiserum against 17-OHP-3-CMO-BSA as was used in the EIA.

Extraction of 17-OHP from Plasma Sample——A plasma sample (20 μl) was diluted with water (0.5 ml), extracted with CH_2Cl_2 (5 ml) using a Vortex type mixer and transferred to another test tube. After evaporation of the solvent under a stream of nitrogen gas, the residue was redissolved by adding 0.05 M PBS–BSA (1 ml) and 0.1 ml of the resultant solution was subjected to EIA.

Ether Extraction of 17-OHP from Dried Blood Filter Paper Disc——One disc of filter paper carrying dried blood was transferred to a test tube (50 mm × 6 mm, i.d.) containing water (0.5 ml) and incubated overnight at room temperature. 17-OHP released into the water was extracted with ether (2 ml) by mixing for 2 min. The water layer was discarded and the ether layer was washed with water (0.5 ml). The ether extract (1 ml) was then transferred to another test tube and evaporated to dryness under a nitrogen gas stream. The resultant residue was subjected to the EIA described above.

Results

Conjugation of 17-OHP with HRP

The 17-OHP-HRP conjugate prepared by the mixed anhydride method was purified by dialysis against 0.05 M PBS followed by gel chromatography on a Bio-Gel P 200 column. The fractions from No. 13 to 16 showed both immunological and enzyme activity. Based on the

spectrophotometric method, the binding ratio of 17-OHP to HRP in the conjugate of fraction No. 15, which showed the highest activity, was determined to be about 2 to 1. This fraction was used for this study.

Dilutions of Anti-17-OHP Antiserum, Second Antibody and 17-OHP-HRP Conjugate

To establish the optimal conditions for EIA, it was necessary to determine the dilutions of anti-17-OHP antiserum, second antibody and 17-OHP-HRP conjugate which would yield optimal fluorescence intensity and still be sensitive to low concentrations of 17-OHP. The fluorescence intensity at B_0 decreased with increasing dilution of anti-17-OHP antiserum. On the other hand, the sensitivity of the assay increased with increasing dilution of anti-17-OHP antiserum; its precision decreased because of a decrease in the amount of 17-OHP-HRP conjugate bound to the DASP beads. Based on a balance between sensitivity and precision, 8×10^4—16×10^4-fold dilutions of anti-17-OHP antiserum were used for the assay.

The optimal concentration of purified second antibody (goat anti-rabbit IgG) for coating the polyacetal beads was examined by varying the concentrations (5.5, 11, 22, 44, 88, 176, and 350 μg/ml) at three dilution levels of anti-17-OHP antiserum (2×10^4, 4×10^4 and 8×10^4). The fluorescence intensity at B_0 reached a plateau at the antibody concentration of 22 μg/ml. Therefore, this purified second antibody solution was used in preparing the DASP beads.

The effect of the 17-OHP-HRP conjugate concentration on the sensitivity of the assay was also examined by varying the dilutions (5, 10, 20, 40, and 80×10^3). Although increasing the dilution of 17-OHP-HRP conjugate solution improved the sensitivity, the fluorescence intensity at B_0 decreased, as did the precision of the assay. Based on a balance between sensitivity and precision, a 2×10^4-fold dilution of 17-OHP-HRP conjugate was chosen for the assay.

Standard Curve of 17-OHP

Figure 1 shows typical standard curves of 17-OHP, constructed for 17-OHP concentrations of up to 100 pg/assay tube. The dotted line is the standard curve of 17-OHP obtained by adding one disc of 17-OHP-free dried blood (3 mm i.d.) at each of the indicated 17-OHP concentrations. The solid line was obtained when the buffer system without a dried blood disc was used. The coefficients of variation for curves A and B range from 2.3 to 4.2% ($n = 6$) and from 2.1 to 6.4% ($n = 6$), respectively. The detection limit of this assay was 1 pg/assay tube. Since one disc of dried blood sample was used in the assay, this value translates into a determination limit of approximate 28 ng/dl of whole blood, or 56 ng/dl of
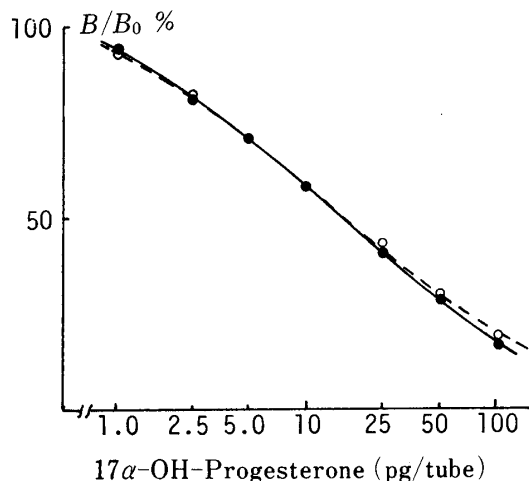

Fig. 1. Standard Curves of 17α-Hydroxy-progesterone
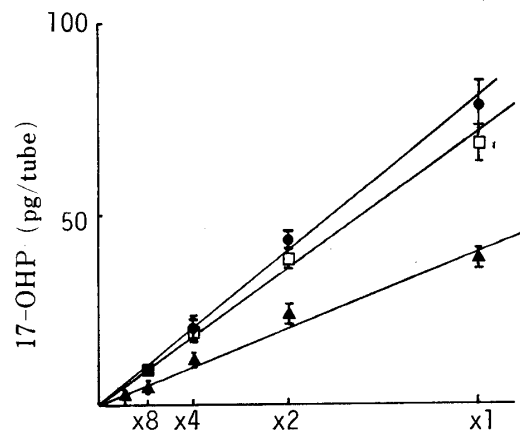
—O—, dried blood system; —●—, buffer system.

Fig. 2. Dilution Curves for Dried Blood Discs

serum, because one disc contained the equivalent of $3.5 \mu l$ of whole blood.[5]

Specificity

The results of the cross-reaction study are illustrated in Table I. The % cross-reaction was calculated at 50% displacement of the 17-OHP-HRP conjugate and [^{3}H]-labeled 17-OHP in EIA and RIA, respectively. The steroids tested were chosen mainly on the basis of their structural similarity and their possible presence in biological fluids. As shown in this table, the relative cross-reactivities of anti-17-OHP antiserum obtained in EIA and RIA were quite similar.

Recovery of 17-OHP from Plasma Samples

The percent recovery was determined by adding known amounts of 17-OHP to plasma samples containing endogeneous 17-OHP. As shown in Table II, at 5 and 10 ng/ml 17-OHP, recoveries of $92 \pm 7.5\%$ and $103 \pm 7.6\%$ ($n=5$) were obtained, respectively.

Elution Efficiency of 17-OHP from Dried Blood on Filter Paper

One disc of dried blood filter paper prepared by adding [^{3}H]-17-OHP solution

TABLE I. Specificity of Antiserum against 17α-Hydroxyprogesterone

Steroid	Cross-reactivity (%) of steroids	
	EIA	RIA
17α-Hydroxyprogesterone	100 %	100 %
Progesterone	4.83	7.90
17α-Hydroxypregnenolone	4.34	6.30
11-Deoxycortisol	2.78	0.90
21-Deoxycortisol	2.50	1.30
Pregnenolone	0.66	—[a]
Deoxycorticosterone	0.15	0.70
16α-Hydroxyprogesterone	0.05	2.50
20α-Dihydroxyprogesterone	0.03	3.20
Cortisone	0.02	—
Cortisol	0.02	0.01
Prednisolone	<0.01	—
Corticosterone	<0.01	—
Tetrahydrocortisol	<0.01	—
17α-Hydroxypregnenolone-3-sulfate[b]	10.0	—
Pregnenolone-3-sulfate[c]	1.49	—
5β-Pregnane-3α,20α-diol-20-sulfate[d]	<0.01	—
5β-Pregnane-3α,20α-diol-3-sulfate[d]	<0.01	—
5β-Pregnane-3α,20α-diol disulfate[e]	<0.01	—
Dehydroepiandrosterone sulfate[c]	<0.01	—

a) No data. b) Triethylammonium salt. c) Potassium salt.
d) Sodium salt. e) Di-potassium salt.

TABLE II. Recovery of 17α-Hydroxyprogesterone from Plasma

Added (ng/ml)	Found (ng/ml)	CV (%)	n	Recovery (%)
—	1.76 ± 0.17	9.7	6	—
5.0	6.35 ± 0.38	6.0	6	91.8 ± 7.6
10.0	12.10 ± 0.76	6.3	6	103.0 ± 7.6

$(4 \times 10^5 \, \text{dpm/ml})$ was eluted overnight at $4 \, ^\circ\text{C}$ with $0.5 \, \text{ml}$ of PBS. The recovery of $[^3\text{H}]$-17-OHP ranged from 95 to 105%.

Precision

Dried blood samples containing various 17-OHP concentrations were examined to assess intra- and interassay variations in the direct EIA method. The intra-assay coefficients of variation for two different samples containing $6.9 \pm 0.5 \, \text{pg/disc}$ $(CV\% = 7.0\%, \, n = 6)$ and $18.2 \pm 1.0 \, \text{pg/disc}$ $(CV\% = 5.4\%, \, n = 6)$ were as indicated in parentheses. The interassay variations were established based on daily duplicate determinations of the 17-OHP concentration in two different specimens with low and high 17-OHP concentrations, i.e. $11.2 \pm 1.6 \, \text{pg/disc}$ $(CV\% = 14.3\%, \, n = 4)$ and $28.5 \pm 2.4 \, \text{pg/disc}$ $(CV\% = 8.3\%, \, n = 4)$.

The precision of the direct EIA method is thus adequate and similar to that of RIAs for 17-OHP in dried blood samples.

Effect of Dilution on Dried Blood Samples

The effect of dilution of dried blood samples was investigated to assess the accuracy of the direct assay without ether extraction. Extracts obtained from three different dried blood samples containing different amounts of 17-OHP were diluted serially with the extract from 17-OHP free dried blood discs and their 17-OHP levels were then determined by the present EIA method. As shown in Fig. 2, linear relationships, which passed through the origin, were observed between the dilution and the 17-OHP concentration.

Comparison of 17-OHP Values Obtained by EIA and RIA

When the 17-OHP values in plasma samples to which 17-OHP had been added were compared, we found a good correlation between the EIA method and the RIA method using
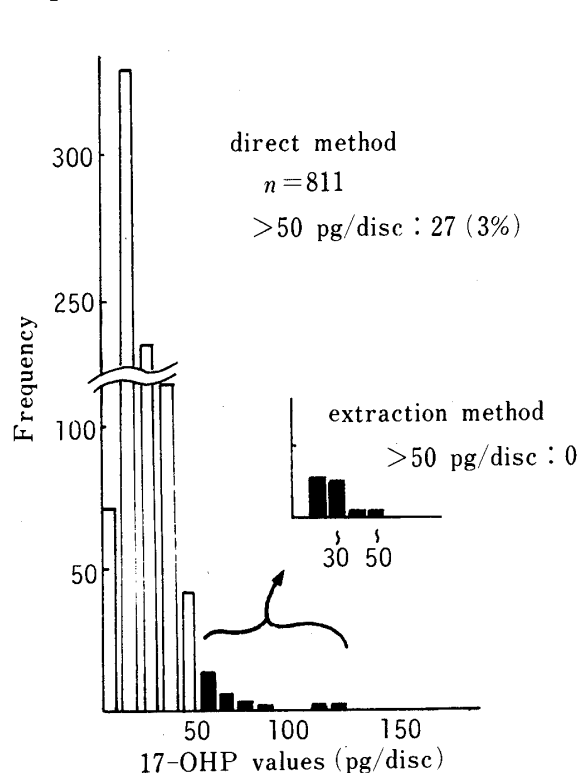

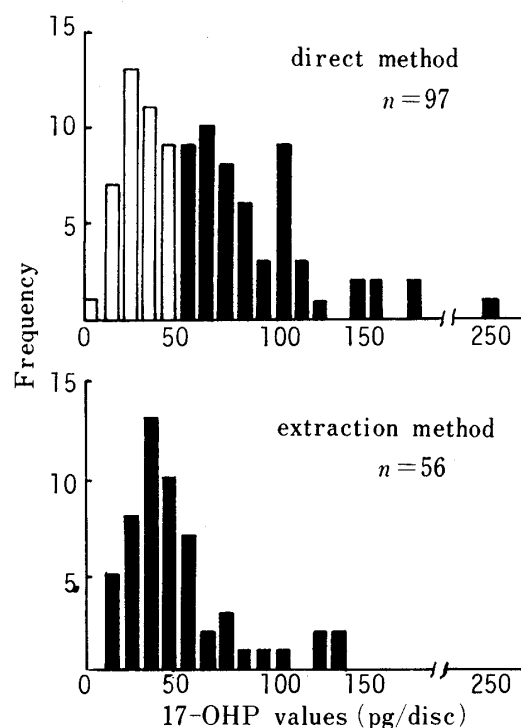Fig. 3. Distribution of 17α-Hydroxyprogesterone in Dried Blood Samples of Newborn Babies

Fig. 4. Distribution of 17α-Hydroxyprogesterone in Dried Blood Samples of Premature Babies

The samples giving values of over 50 pg/disc (■ in the upper figure) were retested by the ether-extraction method.

dichloromethane extraction. The regression line was Y (RIA) $= 0.98$ X (EIA) $- 1.0$ ($r = 0.96$, $n = 33$). The 17-OHP values in dried blood samples determined by the direct EIA method were also compared with the plasma 17-OHP values obtained by the RIA method with ether extraction. There was a high correlation ($r = 0.92$) between the results of the two methods; the regression line was Y (RIA) $= 0.93$ X (EIA) $+ 0.97$ ($n = 27$).

Preliminary Application to Mass-Screening Tests

Duplicate dried blood samples ($n = 811$) from newborns were subjected to the EIA with or without ether extraction. The distribution patterns are shown in Fig. 3.

Discussion

In CAH patients, the blood levels of 17-OHP are extremely high compared to those of normal subjects.[1-3] Mass screening tests for CAH have been developed.[4-6] However, while they are simple and sensitive, they involve RIA with its inherent radiation hazard and disposal problem. There is evidence that EIA is sensitive and reliable.[9] Kohen et al.[10] reported an EIA for 17-OHP which is based on antibody-enhanced hydrolysis of a steroid-umbelliferone conjugate. Although their assay is very convenient since it is homogeneous and does not require the use of isotopes, it is not useful for obtaining a clinical diagnosis of CAH because its sensitivity is low compared to the corresponding RIA. Recently, Hubl et al.[12] reported an EIA of 17-OHP in plasma, blood on microfilter paper and saliva of newborns, children and patients with CAH. In their method, peroxidase was used as the label and the enzyme activity after separation of bound and free fractions by the double antibody-polyethylene glycol method was determined colorimetrically. Although the sensitivity of their method is sufficient to determine 17-OHP in dried blood sample, they used dried blood on a filter paper disc (10 mm in diameter) for the assay. Moreover, an extraction step with methylene chloride and a centrifugation step to precipitate the bound fraction were needed.

We have developed a new chemiluminescence EIA of 17-OHP using glucose oxidase as the label enzyme and the chemiluminescence reaction of bis(2,4,6-trichlorophenyl)oxalate-fluorescent dye.[13] The sensitivity of this method is high, but the measurement of chemiluminescence is difficult for routine mass-screening assay. Therefore, in the present investigation, we selected a fluorophotometric method to assess the enzyme activity rather than a chemiluminescence reaction. Zaitsu and Ohkura[14] developed a very rapid and sensitive method for the fluorimetric assay of peroxidase using 3-(p-hydroxyphenyl)propionic acid (HPPA), which was the best substrate among the various p-hydroxyphenyl compounds examined. We have used HPPA as a substrate for peroxidase assay in developing highly sensitive EIAs for thyroid-stimulating hormone[11] and thyroxine[15] in dried blood samples on filter paper. In the present study, we also employed HPPA as a substrate for the peroxidase assay. The lowest amount of 17-OHP detectable under the optimal conditions was 1 pg/assay tube, corresponding to 2.5 fmol. The sensitivity of the present EIA method is higher than that of the corresponding RIA in the range from 2 pg to 10 pg/assay tube.

Phosphate buffer extraction facilitated the recovery of [^{3}H]-17-OHP from dried blood samples, so we chose the direct extraction method described in "Materials and Methods" for the assay of 17-OHP in dried blood samples. As shown in Fig. 2, a linear relation which passed through the origin was observed between the dilution of extracts from dried blood samples and the 17-OHP concentration. This suggests that there was little interference in the determination of 17-OHP in dried blood samples. The present method makes it possible to perform the extraction directly in the assay tube, using phosphate buffer only. Thus, we have eliminated the necessity for separating the aqueous and organic phases, transferring the organic phase to assay tubes and evaporating the organic solvent. This results in simplification

of the technique and curtailment of possible manipulation errors.

To compare the results of the EIA with those of RIA employing dichloromethane extraction, we assayed 33 plasma samples by both methods. The correlation between the two methods was good; correlation coefficient $r = 0.96$. Dried blood samples from the same subjects were also assayed by the direct EIA method. The results correlated well with the RIA values of the plasma samples; correlation coefficient $r = 0.91$.

Preliminary results of a mass-screening test for CAH using the EIA method with and without ether extraction are shown in Figs. 3 and 4. These samples were assayed in duplicate. The correlation coefficients of duplicate assays ranged from 0.70 to 0.93 and the average was 0.81. The reproducibility is sufficient for a mass-screening test. Solyom et al.[7] reported that in healthy 1- to 5-d-old newborns the highest 17-OHP value was 84 nmol/ml ($= 49.9$ pg/disc). At the International Conference on Neonatal Screening (16—21/August, 1982, Tokyo), Pang et al.[16] reported that in a control population of 4569 consecutive normal neonates ages 2—14 days, the highest value of 17-OHP was 40 pg/disc and the range of values for 20 newborns with proven CAH was 57—980 pg/disc. Shimozawa et al.[17] also reported a pilot neonatal mass-screening in Japan in which the 17-OHP value was 19.2 ± 9.28 pg/disc ($n = 7550$). Therefore, 50 pg/disc was chosen as a cut-off level in this study.

As shown in Fig. 3, 27 cases (3.0%) showed 17-OHP levels in excess of 50 pg/disc. These samples were retested by the ether extraction method. All retested samples showed less than 50 pg/disc.

Figure 4 shows the values of 17-OHP concentration in dried blood samples of prematures ($n = 97$). These samples were collected during the first 4 days of life. Fifty six samples over 50 pg/disc were retested by the ether-extraction method. Their 17-OHP values decreased, but 29 samples still gave values above 50 pg/disc. The mean 17-OHP level of prematures was considerably higher and the distribution pattern was broad as compared with the results for normal newborns. Solyom et al.[7] showed that in stressed premature newborns the 17-OHP values were somewhat higher, especially during the first 3 postnatal days. These results may be attributed to the fact that prematures themselves have a high 17-OHP level and the antiserum against 17-OHP used in the assay cross-reacted with water-soluble steroids in premature blood, e.g. sulfates or glucuronides of steroids structurally related to 17-OHP. As shown in Table I, water-soluble 17α-hydroxypregnenolone-3-sulfate showed 10% cross-reactivity. Therefore, the more specific antiserum should be used for the direct EIA. The 17-OHP values of two CAH patients were 179 ng/ml of serum ($= 322$ pg/disc) (63 d after birth) and more than 200 ng/ml of serum ($= 360$ pg/disc) (97 d after birth). These values decreased to 56 ng/ml of serum ($= 100$ pg/disc) and 208 ng/ml of serum ($= 374$ pg/disc) by the ether-extraction method, but the values were invariably higher than the upper limit for normal newborns illustrated in Fig. 3.

In conclusion, our EIA method is superior to previous IRAs.[4-7] because of its high sensitivity, good reproducibility, the absence of radiation hazard and its use of less expensive apparatus. For the purpose of mass screening for CAH, the direct EIA method without ether extraction can be performed and samples giving over 50 pg/disc should be retested and further examined for plasma 17-OHP.

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