Enzyme-Linked Immunosorbent Assay for 17α-Hydroxyprogesterone in Dried Blood Spotted on Filter Paper

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In this rapid, cost-effective, enzyme-linked immunosorbent assay for 17α-hydroxyprogesterone (17-OHP) eluted from dried blood spotted on filter paper, second antibody is coated onto the microwell plate and horseradish peroxidase (EC 1.11.1.7) is the label enzyme. Antiserum to 17-OHP was prepared by using 4-(2-carboxyethylthio)-17-OHP-bovine serum albumin conjugate as immunogen. Enzyme conjugate was prepared from 4-(2-carboxymethylthio)-17-OHP and peroxidase. The blood spots are assayed in the microwells without extraction or centrifugation steps. The detection limit of the assay is 1 μg/L, equivalent to 3.5 pg (10.6 fmol) per disc. Intra- and interassay CVs at two steroid concentrations (7.38 and 22.79 μg/L) ranged from 3.74 to 11.90% (n = 5), and 9.49 and 9.83% (n = 5), respectively. Results correlated well (r = 0.91) with those of a fluorescence enzyme immunoassay. The sensitivity, specificity, and precision of this method made it potentially useful in the mass screening of neonates for congenital adrenal hyperplasia.

Additional Keyphrases: screening * newborns * heritable disorders * congenital adrenal hyperplasia * enzyme-linked immunosorbent assay

Congenital adrenal hyperplasia (CAH) is an inborn error of metabolism caused by various enzymatic defects of adrenal steroidogenesis. In the most common type, the result of deficiency of steroid 21-hydroxylase (EC 1.14.99.10), the 17-hydroxyprogesterone (17-OHP) concentration is markedly increased in the blood of untreated patients. Thus, the concentration of this steroid in blood is commonly measured, to aid in the rapid diagnosis of CAH and for monitoring the effectiveness of therapy in neonates. Most recent radioimmunoassays of 17-OHP are sensitive, but involve an extraction with organic solvent (1-4), making this technique unsuitable for use in large-scale screening. Wallace et al. (5, 6) and Hofman et al. (7) reported the use of direct RIA for 17-OHP in blood spots, with a radioactive iodinated 17-OHP tracer. Huble et al. (8) reported an enzyme immunoassay of 17-OHP in plasma or dried blood on a paper disc and in saliva of newborns, children, and patients with CAH. However, their method was insufficiently sensitive to detect the 17-OHP in a 3-mm (diameter) dried blood disc. Moreover, extraction with methylene chloride and centrifugation were needed, to precipitate the bound fraction. Klingler et al. (9) developed a chemiluminescent immunoassay of 17-OHP with isoluminol derivative as a label and applied this to mass-screening of neonates for CAH. Elsewhere we have reported our development of a chemiluminescence enzyme immunoassay with glucose oxidase (EC 1.1.3.4) and a fluorescence enzyme immunoassay with horseradish peroxidase (EC 1.11.1.7) (10, 11). Although chemiluminescence enzyme immunoassay is highly sensitive in comparison with RIA, the technique is too cumbersome for use in routine mass-screening. Our fluorescence enzyme immunoassay of 17-OHP (11) has been used since 1982 in several screening laboratories in Japan, but it has an unacceptably high false-positive rate for premature infants because the antiserum cross reacts with several steroids in such samples (12). Also, the separation technique, which involves double-antibody-coated beads and fluorophotometry, is troublesome for mass screening tests.

For the present study we have prepared a specific anti-17-OHP antiserum by using as immunogen 4-(2-carboxyethylthio)-17α-hydroxyprogesterone (4-CET-17-OHP) conjugated with bovine serum albumin. Using this antibody, we have developed a simple and sensitive enzyme-linked immunosorbent assay (ELISA) system for 17-OHP eluted from dried-blood samples on discs of filter paper.

Materials and Methods

Apparatus

ELISA plates ("Microwell plate 96F without LID-PS-SH") were obtained from Nunc Co., DK 4000 Roskilde, Denmark. Plastic film "MS-300," used to cover the plates, was from Sumitomo Bakelite Co., Tokyo. Eight-channel variable-volume pipettes, a shaker, and an semi-automatic microtiter plate reader "Titertek Uniskan," and a 12-channel semi-autoaspirator "Nunc-Immu Wash 12" were all from Flow Laboratories, Irving, Finland. The programmable NEC PC 9801F computer was from Nippon Electric Co., Tokyo, Japan.

Reagents

17-OHP and other steroids were purchased from Sigma Chemical Co., St. Louis, MO. 2,2'-Azino-di(3-ethylbenzthiazoline sulfate) (ABTS®) was from Boehringer Mannheim-Yamanouchi Co., Tokyo, Japan. Horseradish peroxidase (261 kU/g) was from Toyobo Co., Osaka, Japan, Toyopeal HW 55 SF from Toyo Soda Co., Tokyo, Japan. All other chemicals and buffer salts were analytical grade.

To coat the plates, we used sodium carbonate buffer (50 mmol/L, pH 9.5). The post-coating solution contained, per liter, 10 g of bovine serum albumin and 0.5 g of sodium azide. Assays were carried out in phosphate buffer (0.1 mmol/L, pH 7.4) containing, per liter, 27 mmol of potassium chloride, 1.37 mmol of sodium chloride, and 5.0 mL of Tween 20 polyoxyethylene (20) sorbitan monolaurate, which was diluted 10-fold with distilled water before use.

The substrate solution (pH 4.0) contained 2.5 mmol of ABTS, 5.0 mmol of H2O2, 300 mmol of citric acid, and 51 mmol of disodium hydrogen phosphate per liter. Sodium azide solution (1 g/L) was used to stop the enzyme reaction.
Samples

17-OHP-free serum was prepared by treating pooled human serum with charcoal (13). Erythrocytes in heparinized blood from apparently normal subjects were washed twice with isotonic saline, once with 17-OHP-free serum, and then recombined with 17-OHP-free serum to adjust the hematocrit to 55%, to mimic the higher hematocrit in newborns. To this material we added known amounts of 17-OHP to give final concentrations of 0, 1.25, 2.50, 6.25, 12.5, 25.0, 62.5, 125, and 250 μg/L in whole blood. These 17-OHP blood standards were then spotted on filter-paper cards used in infant screening (Toyo Filter Paper Co., Tokyo, Japan), to serve as standards for the dried-blood discs.

Procedure

Antiserum preparation. Antiserum to 17-OHP was raised in rabbits by using 4-CET-17-OHP–albumin conjugate as immunogen, prepared as described previously (10). This antiserum is highly specific, as characterized by RIA (14). Before use, we diluted the anti-17-OHP serum 80 000-fold with the assay buffer. The second antibody was goat antirabbit IgG (affinity-purified IgG fraction, 4.1 g/L) from Miles–Yeada Co., Israel.

Enzyme conjugate. We prepared 17-OHP-HRP conjugate by the mixed-anhydride method as previously described (10).

Coating of second antibody to the microwell plates. Microwell plates were coated by adding 200 μL per well of 20 mg/L goat antiserum to rabbit IgG, a solution in coating buffer. The plates were kept overnight at 4 °C. The IgG solution was removed and any binding sites were blocked by adding to each well 300 μL of a 10 g/L bovine serum albumin solution containing 0.5 of sodium azide per liter. The coated plates, covered with the plastic film, were stable for at least two to three months at 4 °C. Before use, each plate was washed with 300 μL of assay buffer per well and excess fluid removed by tapping the inverted plate on a paper towel.

Direct assay. The discs (3 mm in diameter) were punched out from spots of standard or dried-blood sample on filter paper cards, allowed to fall directly onto the appropriate wells of the plate, followed by 100 μL of assay buffer, 50 μL of 1:80 000 diluted anti-17-OHP antiserum, and 50 μL of 1:3000 diluted 17-OHP-HRP conjugate. The plate was covered with the plastic film, shaken by swirling, and left overnight at 4 °C. The contents of the plate were then aspirated and the wells were washed three times with assay buffer by use of the 12-channel aspirator. The plate was then inverted over a paper towel. To each well, 150 μL of the ABTS–H₂O₂ substrate solution was added, and the color was allowed to develop in the dark for 2 h at room temperature. Then 50 μL of a 1 g/L sodium azide solution was added to terminate the enzymatic reaction, and absorbances were measured at 420 nm with a semi-automatic microplate reader. All standards and samples were run in duplicate. The computer evaluated and edited the data, plotted the standard curve, and calculated the assay statistics and the concentration of 17-OHP in dried blood discs.

Extraction assay. Samples with high values for 17-OHP, as detected by the direct assay, were confirmed by the extraction assay. One disc was placed in glass culture tube and eluted for 2 h at room temperature by addition of 500 μL of isotonic saline. To extract steroids we used 1 mL of diethyl ether and vigorous shaking on a vortex-mixer for 1 min. The tube was dipped into solid CO₂-acetone to freeze the water layer. The ether extract was transferred to another tube, evaporated under nitrogen, and the residue was dissolved in 100 μL of assay buffer. Fifty microliters of the resulting solution was then subjected to the ELISA as outlined for the direct assay.

Fluorescence ELISA of 17-OHP. The previous method (10) was used for comparison with the ELISA method.

Preliminary application to mass-screening. Duplicate dried-blood samples obtained from 5558 neonates (ages five to seven days) were assayed by the ELISA, either the direct or the extraction assay.

Results

Optimal assay conditions. As a compromise between sensitivity and precision, we used an 800 000-fold dilution of the antiserum to 17-OHP in the assay. Increasing the dilution of the 17-OHP–peroxidase conjugate solution from 1500- to 5000-fold improved assay sensitivity, but the absorbance at B₀ decreased, as did the precision of the assay. Therefore, we used 3000-fold-diluted 17-OHP–peroxidase conjugate solution in all subsequent assays.

Standard curves. Figure 1 shows typical standard curves for 17-OHP in dried blood discs, for both the direct assay and the extraction assay. The CV for each point ranged from 3.78 to 10.70% (mean 6.60%) for the direct assay, 5.40 to 11.60% (mean 8.80%) for the extraction assay. The limit of detection for 17-OHP (i.e., the response distinguishable from that for zero 17-OHP) was 1 μg/L. Intra- and interassay CVs were evaluated by testing five replicates each of samples with low and high 17-OHP concentrations in dried blood: samples A (7.38 μg/L) and B (22.79 μg/L). Intra-assay CVs ranged from 3.80 to 11.90% (n = 5) and 3.74 to 10.45% (n = 5) for samples A and B. Interassay CVs were 9.49 and 9.83% (n = 5) for samples A and B.

Correlation with the fluorescence enzyme immunoassay for 17-OHP. Using the present ELISA (y) and the fluorescence enzyme immunoassay for 17-OHP (x) reported in the previous paper (10), we assayed, in duplicate, 80 samples of dried blood from neonates. The linear correlation coefficient between the 17-OHP values as determined by those two methods was 0.910, the slope 0.789, and the y-intercept -1.940.

Antibody specificity. We evaluated the specificity of the antiserum we prepared by assaying potentially cross-reacting steroids. Table 1 gives the results.

Application to mass screening. Results of preliminary application to mass screening of CAH are shown in Figure 2. In the direct assay, the mean value for 17-OHP was 21.9 μg per liter of blood. Values for 630 samples exceeded 36 μg/L.
Table 1. Cross Reactivity of Some Steroids with Rabbit Antiserum to 4-(2-Carboxyethylthio)-17α-hydroxyprogesterone–Albumin in the ELISA

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross reactivity, %</th>
</tr>
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<tbody>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>100.0 (100.0)</td>
</tr>
<tr>
<td>17α-Hydroxyprogrenenolone-3-sulfate</td>
<td>5.54 (10.0)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.13 (4.83)</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.72 (0.02)</td>
</tr>
<tr>
<td>17α-Hydroxyprogrenenolone</td>
<td>0.51 (0.34)</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>0.05 (0.66)</td>
</tr>
<tr>
<td>16α-Hydroxyprogesterone</td>
<td>0.05 (0.05)</td>
</tr>
<tr>
<td>16α-Hydroxyprogrenenolone</td>
<td>0.01 (0.01)</td>
</tr>
<tr>
<td>21-Deoxycortisol</td>
<td>0.01 (2.50)</td>
</tr>
<tr>
<td>Androsterone</td>
<td>0.01 (--)</td>
</tr>
<tr>
<td>11-Deoxyandrocorticosterone</td>
<td>0.01 (2.76)</td>
</tr>
</tbody>
</table>
| 20α-Hydroxypregnen-3-one | 0.06 (--)
| 5β-Pregn-3α,20α-diol-20-sulfate | 0.01 (0.01) |
| 5β-Pregn-3α,20α-diol-disulfate | 0.01 (0.01) |

In parentheses: cross reactivity in the fluorescence enzyme immunoassay (10).

Fig. 2. Distribution frequency of 17α-hydroxyprogesterone concentration in dried blood discs as determined by use of the ELISA corresponding to 50 pg per disc. We examined these by the extraction assay. As shown in the inset to Figure 2, the values were lower than those obtained in the direct assay, the mean 17-OHP value for these samples being 2.1 μg/L.

Discussion

The specificity of an antiserum for steroids is markedly influenced by which position on the steroid molecule is used for conjugation to the carrier protein. Hosoda et al. (15) suggested that conjugation at carbon 4 is effective in preparing specific antiserum against testosterone or cortisol. Guided by this, we used a C-4 conjugate as the immunogen in this study. Cross reactivities to some steroids that are similar to 17-OHP in structure or are present in abnormally high concentrations in the blood of neonates were measured by the fluorescence enzyme immunoassay and compared with the cross reactivity of the previous anti-17-OHP antiserum, which was prepared by using 3-carboxymethyl oxime-17-OHP–bovine serum albumin (10). As shown in Table 1, the specificity of the antiserum used here was superior to that of the previous antiserum prepared by using a carbon-3 derivative.

The sensitivity of enzyme immunoassays for steroids is affected by the combination of antibody and enzyme-labeled steroid derivative, that is, whether it is a "homologous" or "heterologous" system. Van Weemen and Schuurs (16) suggested that the sensitivity of enzyme immunoassay for estrogen could be considerably improved by using heterologous systems. Hosoda et al. (17) systematically examined many steroid derivatives and antisera against them and reached the same conclusion, as did we for cortisol (18), dehydroepiandrosterone (19), and thyroxin (20). Therefore, we examined homologous and heterologous systems of 17-OHP derivatives: 3-O-carboxymethyl oxime-, 4-carboxymethylthio-, 4-carboxyethylthio-, 11-hemisuccinoy-, and the C-7 derivative (obtained from Miles Co.) (14). Among them, the bridge heterologous combination, involving antiserum prepared by using 4-carboxyethylthio-17-OHP–bovine serum albumin and 4-carboxyethylthio–horseradish peroxidase conjugate, was the most sensitive enzyme immunoassay for 17-OHP. The bridge heterologous system, in which a shorter bridge is used in enzyme labeling than that in the homologous or the longer bridge system, can increase the sensitivity because antibody and labeled enzyme lower the affinity of antibody for the antigenic determinant in the labeled antigen (17). Guided by the previous results, we used the bridge heterologous system in this ELISA method.

In general, the colorimetry is less sensitive than the fluorometric method, but the sensitivity of the present ELISA is almost the same as that of the previous fluorescence enzyme immunoassay in which peroxidase was used as the label (10). The detection limit of the present ELISA is 1 μg/L, equivalent to 3.5 pg (10.6 fmol) per disc if the volume of blood in one 3-mm disc is calculated to be 3.6 μL. This sensitivity suffices for use in mass screening for CAH, because the cutoff value for CAH proposed by Fang et al. (21) is 50 pg per disc.

Values for 17-OHP obtained by the ELISA (x) and the fluorescence enzyme immunoassay (y) correlated well: y = 0.78x − 1.94; r = 0.910.

To assess the usefulness of this ELISA for CAH screening, we assayed samples from two CAH patients and five samples from premature infants by the direct assay and the extraction assay. As shown in Table 2, the 17-OHP values for the patients were 452.4 and 93.5 μg/L by the direct assay and—still showing high values—214.7 and 51.2 μg/L by the extraction assay. On the other hand, the 17-OHP values for the premature babies are high by the direct assay, over 50

Table 2. 17α-Hydroxyprogesterone Concentrations In Dried-Blood Samples from Congenital Adrenal Hyperplasia Patients and Premature Infants

<table>
<thead>
<tr>
<th>Baby</th>
<th>Direct assay</th>
<th>Extraction assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>452.4</td>
<td>214.7</td>
</tr>
<tr>
<td>B</td>
<td>93.5</td>
<td>51.2</td>
</tr>
<tr>
<td>C</td>
<td>90.0</td>
<td>4.9</td>
</tr>
<tr>
<td>D</td>
<td>17.1</td>
<td>4.0</td>
</tr>
<tr>
<td>E</td>
<td>64.4</td>
<td>4.1</td>
</tr>
<tr>
<td>F</td>
<td>105.8</td>
<td>2.6</td>
</tr>
<tr>
<td>G</td>
<td>112.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>

A, B: patients with congenital adrenal hyperplasia; C-F: premature.
pg/disc, but normal by the extraction assay. Therefore, we can detect CAH in a neonate by the combination of direct assay and extraction assay.

As shown in Figure 2, 630 samples with high 17-OHP concentrations by the direct assay showed normal values by the extraction assay. Closely related water-soluble steroids, such as 17-hydroxyprogrenenolone-3-sulfate, probably are responsible for this discrepancy. Although such steroids cross react with the anti-17-OHP antiserum used in this study only to a small extent, as illustrated in Table 1, they are present in exceptionally high concentrations during the neonatal period of premature babies. Those steroids could be eliminated by extraction with ether. The cutoff value for 17-OHP to be used in screening for CAH will be decided by the specificity of antiserum used in the assay. We are doing a large-scale preliminary mass-screening test to decide the cutoff value for the direct assay presented here.

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References


