Direct Solid-Phase Radioimmunoassay for Screening 17α-
hydroxyprogesterone in Whole-Blood Samples from Newborns

Jndaay F. Hofman, James E. Klainiecki, and Elizabeth K. Smith

We describe a direct, solid-phaseRIA for 17α-hydroxyprogesterone (17-OH-P) that we are using to screen neonates or congenital adrenal hyperplasia. Phosphate buffer containing danazol and anti-17-OH-P is placed in tubes coated with antibody to IgG. The tubes also contain standards, controls, and blood samples on filter paper discs 3 mm in diameter. 125I-labeled 17-OH-P is added to each tube. The mixture is vortex-mixed and incubated overnight. The fluid and disc are removed, the radioactivity remaining in the tubes is counted, and the amount of 17-OH-P per disc is calculated by using a 3g-logit transformation of the standard curve. Results compare favorably with those by two extraction assays. Inter- and intra-assay CVs were <11% and <9%, respectively. Sensitivity was 2 pg per assay tube. There is no significant cross-reactivity with structurally related steroids at their physiologic concentrations. Analytical recovery of added 17-OH-P averaged 104%. 17-OH-P in whole blood spotted on filter paper is stable for at least six months.

Additional Keyphrases: steroids • congenital adrenal hyperplasia • radioimmunoassay • blood samples on filter paper • heritable disorders • cutoff value

Congenital adrenal hyperplasia (CAH) is caused by a deficiency of the enzyme 21-hydroxysteroid dehydrogenase NADPH (EC 1.1.1.151), which converts 17α-hydroxyprogesterone (17-OH-P) to 11-desoxycortisol in the synthesis of cortisol. This disorder may account for 90 to 96% of inborn errors of the adrenal steroid biosynthetic pathway (1). Diagnosis of the disease by measurements of 17-OH-P in serum is well established (2). The incidence of the disease in the U.S. population is unknown. However, a retrospective study in Europe (3) and small prospective studies in Europe (4), Asia (5), and Alaska (6) suggest that it exceeds 1:10,000. A rate generally considered cost effective for neonatal screening. CAH also fulfills other criteria suggested for neonatal screening (7): it is treatable with oral medication, an absolute difficulty to recognize in the neonatal period, and requires prompt treatment—in some cases to save lives, in others to allow attainment of normal growth.

For these reasons, recent publications have recommended that infant screening for CAH be instituted if a suitable assay can be developed. This recommendation was submitted by a committee of experts on inborn metabolic disease at the Council of Europe (7), and also by Lebovits et al. (9) upon the completion of their study in which they found increased age at which CAH was diagnosed in 32 children born between 1956 and 1979 was 12.6 months. Previously developed assays (6, 9–11, 17) for CAH screening with filter-paper blood spots are not suitable for use in large-scale screening because of the various combinations of analytical procedures involved: extraction, multiple reagent addition, centrifugation, and addition of liquid scintillation fluid for counting tritium radioactivity. The radioimmunoassay we describe here involves no extraction or centrifugation, and only two reagents are added. In 24 h 1200 samples can be processed by this method.

Materials and Methods

Sampling: We punch out filter paper discs, 3 mm in diameter and saturated with whole blood, with a Phillips Punch Indexer, Model VII (Fundamental Product Co., North Hollywood, CA 91601). The filter-paper screening cards (no. 903; Schleicher & Schuell, Inc., Keene, NH 03451) are those used by the states of Washington and Alaska in screening newborns for hypothyroidism and phenylketonuria.

Chemicals: Radioiodinated 17-OH-P (labeled with 125I at the α-position) and anti-17-OH-P (rabbit) were purchased from Radioassay Systems Laboratories, Inc., Carson, CA 90746. Polystyrene tubes (12 × 75 mm) coated with goat antirabbit IgG were purchased from Micromedic Systems, Inc., Horsham, PA 19044. Sodium phosphate (dibasic, heptahydrate) and EDTA (dissodium salt) were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Other components used in buffer and standard preparations were sodium azide (Eastman Organic Chemicals, Rochester, NY 14650), bovine serum albumin (98–99% albumin, cat. no. A7860; Sigma Chemical Co., St. Louis, MO 63178), Norit A charcoal (Pfanstiehl Laboratories, Waukegan, IL 60085), and absolute ethanol (USP; U.S. Industrial Chemicals Co., New York, NY 10016). Danazol (17α-pregna-2,4-dien-20-yno(2,3)dioxazol-17-ol) was a gift from Sterling Winthrop Research Institute, Rensselaer, NY 12144. Pure 17-OH-P for standards and other steroids used in interference studies were purchased from Steraloids, Inc., Wilton, NH 03086.

Preparation of standards and controls: Collect fresh whole blood in a 7-mL tube containing 10.5 mg of EDTA (dissodium salt). Separate plasma from cells by centrifugation. Wash the cells twice with a volume of isotonic saline equal to the volume of the plasma removed. Centrifuge the cells, discard the supernatant fluid, and determine the hematocrit of the pooled cells.

Add Norit A (40 g/L) to plasma, to remove endogenous steroids. Mix with a magnetic stirrer, refrigerate overnight, and remove the charcoal by multiple centrifugations until no charcoal is visible after centrifugation. Then mix equal volumes of blood cells and plasma to give steroid-free blood with a hematocrit of 50.

To prepare the standards or controls, evaporate an appropriate amount of 17-OH-P solution (100 μg/L in 95% ethanol) in a glass tube. Add the steroid-free whole blood preparation, mix, and allow to equilibrate for at least an hour.

Apply 50-μL aliquots of the same standards at 17-OH-P (10, 20, 40, 74, and 100 μg/L) and for controls at two different concentrations (approximately 22 and 56 μg/L) to the filter-paper cards used in infant screening. Air-dry overnight at room temperature, and then store the cards at 4°C in plastic bags containing desiccant.

Office of Public Health Laboratories and Epidemiology, Division of Health, Department of Social and Health Services, 1409 Smith Tower, Seattle, WA 98104.
Received January 25, 1985; accepted April 2, 1985.
Radioimmunoassay for 17-OH-P: Punch the discs saturated with whole blood from standard, control, or sample cards directly into antibody-coated tubes. To each tube add 1 mL of buffer (pH 7.4; per liter: 63 mmol of phosphate, 25 mmol of NaNO₃, 13 mmol of EDTA, and 3.4 g of bovine serum albumin) containing a 220 000-fold dilution of antibody and 400 ng of danazol (to free 17-OH-P from binding protein). Then add 100 µL (28 nCi) of ¹²⁵I-labeled 17-OH-P in the same buffer, mix, and incubate the tubes overnight at room temperature. Aspirate and discard the liquid and filter-paper discs the next morning and count the bound radioactivity remaining in the tube with a multiple-channel gamma counter. (We used an Apex 10-channel counter from Micromedic, Inc., Horsham, PA, interfaced to a computer from Prime Computer, Inc., Natick, MA 01760, with in-house programming.) After a log-logit transformation of the standard curve, the amount of 17-OH-P per 3-mm disc is calculated. In routine screening, we assay one set of six standards in duplicate and two of each concentration of control for each 180 samples.

Comparison methods: The two comparison assays, described elsewhere (15–17), are both used routinely to diagnose and monitor patients. The high specificity of their antisera has been described (15, 17).

Results

Standard matrix: Figure 1 compares standard curves obtained for 17-OH-P standards in whole blood on filter-paper discs and standards in assay buffer. The curve for the standards in buffer was superimposable on that for a commercially prepared standard in serum (Wien Laboratory, Succasuna, NJ 07876). For routine use, we linearized the curve by log-logit transformation. For data on 39 curves for filter-paper standards, used in 39 routine assays, the regression equation was logit B/B₀ = −0.17 (SD 0.09) log pg per disc + 2.65 (SD 0.19); r = 0.997 (SD 0.005).

Precision: Intra-assay CVs, assessed by measuring 17-OH-P controls at 22 and 56 µg/L, 39 pairs at each concentration (13), were 7.6% and 8.5%, respectively. The corresponding interassay CVs, assessed from one high and one low control in 39 consecutive routine assays, were 8.5% and 10.2%.

Analytical recoveries were determined by adding 17-OH-P to pooled fresh whole blood to give six concentrations (10, 20, 40, 60, 75, 100 µg/L). The samples were spotted on filter paper, dried, and at least four 3-mm discs were assayed for each concentration. Recoveries ranged from 97 to 106% (mean 104%). If the cells were frozen before 17-OH-P was added to the blood, recoveries were consistently less, averaging 61% (range 59–64%) (14).

Linearity. Using steroid-free blood, we made five dilution of blood from a known CAH patient, giving 17-OH-P concentrations ranging from 0 to 114 µg/L. The dilutions were spotted on filter paper cards, dried overnight, and discs were punched out and assayed in duplicate. The linear regression equation summarizing the results (x = calculated value, y = measured value) was y = 0.96x − 1.46 (r = 0.97, n = 10).

Interference was determined by assaying dilutions of steroids similar in structure to 17-OH-P or present in abnormally high concentration in serum. None of the steroids tested (progesterone, 17-hydroxyprogrenolonc, cortisol, androstenedione, dehydroepiandrosterone, 11 deoxycorticisol, 16-hydroxyprogesterone, 16-hydroxydehydroepiandrosterone, and prednisolone) showed notable interference (Table 1).

Sensitivity: The lower limit of detection, calculated from two standard deviations of the count/min for the zero standard (10 tubes), was 2 pg per tube. The concentration a 50% of the zero-standard binding ranges from 28 to 36 µg/L.

Stability: We spotted 10 cards with whole blood to which 17-OH-P was added, and 20 discs were punched from each card. Three cards were stored in a metal cabinet at room temperature, three were stored with desiccant in a plastic bag on an open shelf near windows, two were stored with desiccant at 2°C, and two were stored with desiccant a 35°C. Under all conditions the samples were stable for seven months and only at 35°C and on an open shelf did samples show any instability at nine months (Table 2).

### Table 1. Cross Reactivity of Some Steroids with Rabbit Anti-17α-OH-P-7α-CETA⁺⁻BSA in the Direct RIA

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% cross reactivity*</th>
<th>Infants</th>
<th>Mothers</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-OH-progesterone</td>
<td>100</td>
<td>33 (19)</td>
<td>11 (1)</td>
</tr>
<tr>
<td>17-OH-pregnenolone</td>
<td>2</td>
<td>13 (21)</td>
<td>10 (21)</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>1</td>
<td>8 (22)</td>
<td>9 (22)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1</td>
<td>68 (19)</td>
<td>271 (1)</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.1</td>
<td>104 (19)</td>
<td>548 (1)</td>
</tr>
<tr>
<td>Estriol</td>
<td>&lt;0.1</td>
<td>32 (21)</td>
<td></td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>&lt;0.1</td>
<td>5 (21)</td>
<td>12 (21)</td>
</tr>
</tbody>
</table>

*Carboxyethyl ether.  **Concentration measured/concentration added (average of duplicates at two concentrations).  †Numbers in parentheses refer to literature cited.  ‡Concentration in cord blood.

### Table 2. Stability of 17-OH-P in Blood Spots on Filter Paper

<table>
<thead>
<tr>
<th>17-OH-P concn. µg/L</th>
<th>No. months stored</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>65 b</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
</tr>
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<td>5</td>
<td>64</td>
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<td>6</td>
<td>67</td>
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<td>7</td>
<td>67</td>
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<tr>
<td>8</td>
<td>66</td>
</tr>
<tr>
<td>9</td>
<td>57</td>
</tr>
</tbody>
</table>

*Storage conditions: A = in the dark at room temperature; B = on an open shelf at room temperature; C = with desiccant at 4°C; D = with desiccant 35°C; 0 storage: 65 (SD 4.5) µg/L (n = 10).  †Concns. are average duplicates.
Comparison of methods. Samples from 4339 infants were spotted on ordinary infant-screening cards (four blood spots). One spot was sent to be assayed by a published assay involving an extraction method (17). A disc from another spot was assayed in this laboratory. In both assays all but three samples were within the normal range, and the same three were abnormal in both tests.

We also compared the blood-spot assay with a serum RIA method (15, 16). Blood was collected from known CAH patients under treatment. Both serum and whole-blood spots on filter paper were prepared from each patient's sample and assayed by the appropriate method. Figure 2 shows a graphic comparison of the two methods. Both statistical and visual analyses show the two methods compare favorably, with a correlation coefficient of 0.97 and no samples positive by only one method.

Discussion

Standard matrix: Because of the errors inherent in sampling blood on filter paper discs, liquid standards are used in many infant-screening assays. However, in this assay, the convenience of a filter paper standard, together with the advantage of having standard and sample in the same medium, offers benefits that we believe outweigh the lesser precision. In addition, filter paper standards are stable in the refrigerator for at least six months, so large batches can be made at once. The absolute difference between results on liquid and spot standards is small (Figure 1). If a zero-standard blood disc is added to the liquid standard, the curve becomes superimposable on that for the paper standard, indicating that the slight difference seen is probably ascribable to matrix effects.

Precision: The precision of the assay compares favorably with other screening assays and is adequate for the clinical needs of the test. At the medical decision level, 36 µg/L, a CV of 8.5% gives a range of 27–45 µg/L (p = 0.01). This range does not overlap with known positive results, as all positive 21-hydroxylase-deficient infants have had values exceeding 71 µg/L.

Recovery of added 17-OH-P averaged 104%. Results for dilutions of a CAH patient's blood spotted on filter paper gave a straight line when plotted vs. expected values. In contrast, we found that recoveries from frozen cells were consistently less, averaging 61%. The hemolysis of the cells may cause a different pattern of absorption by the paper. Samples are collected directly from a heel stick onto paper, so this is not a problem in infant screening, but this effect does make it necessary to use fresh cells in preparing standards and controls.

If sample cards are stored at freezing temperatures after the blood is applied and dried, there is no effect on assayed concentration.

Concern for interference in radioimmunoassays centers around structurally similar compounds that may be present in relatively large concentrations. The antibody used in this assay is extremely specific. Even progesterone at the relatively high concentrations present in serum of neonates (68 µg/L) would only give 17-OH-P values of 0.68 µg/L in this assay (19).

Our comparison of methods included two methods that involve extraction. In neither case were results by the direct assay significantly higher. Determination of normal and "presumptive positive" was the same in both screening assays. Results of the direct blood-spot and serum assays also compared well (Figure 2). Most of the variation is in the higher concentrations, where the direct assay is less precise.

Stability: The sample on filter paper is stable under conditions involved in mailing to a central location. Because mailed samples may be exposed to many different conditions, we checked stability under various temperatures and lights. After nine months only the sample exposed to natural light and the sample kept at 35 °C showed sign of deterioration. We keep controls and standards in the refrigerator and samples in drawers at room temperature. Stability under these conditions is good.

Because the question of the duration of stability of samples was discussed at the 1984 National Neonatal Screening Symposium (18), the long-term effects of storage are being investigated with both added 17-OH-P and CAH patients' blood. Cards have been prepared in sufficient quantity to check stability at monthly intervals for eight years. Discs from CAH infants, taken from cards three to five years old, have had values >71 µg/L; thus we expect that the steroid will prove to be stable for long periods of time.

Medical decision level: When a sample value exceeds 36 µg/L, we request a repeat blood-spot specimen. If the value on this sample also exceeds 36 µg/L, we recommend to the patient's doctor that a workup be done to determine if the infant has CAH. We chose this decision level because the literature indicated we would not be missing any positives at this level and because the number of samples needing followup work was appropriately small. In Pang's laboratory (6), which has had the only other American screening experience of this sort, a cutoff value of 41 µg/L was used. Our results are similar to theirs: 36 µg/L equals 50 pg per disc, a convenient number for a decision level and one that gives us a little leeway, because their lowest positive value was 41 µg/L.

The assay has performed well during the four months it has been in use, averaging 10 000 samples a month. It is easy to run and lends itself to the use of either automated or semi-automated pipettors. If samples for which a followup is called, but which are found normal, are considered false positives, the false positive rate is 0.1%. Generally these have been premature or extremely ill infants. No false negatives have been reported.

This work was supported by grant MCJ-530478-02-0 from the U.S. Dept. of Health and Human Services.
References


