Assessment of Corpus Luteum Function by Direct Radioimmunoassay for Progesterone in Blood Spotted on Filter Paper

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In this specific, direct RIA for progesterone in capillary blood dried on filter paper, progesterone is eluted, with phosphate buffer containing bovine serum albumin, from 5.9 μL of blood dried on 5.0-mm (diameter) discs of filter paper. The eluate is assayed, with 125I-labeled progesterone-11α-glucuronyl-tyramine as tracer, with separation by a double-antibody solid-phase technique. The sensitivity of the assay is 4.7 pg per tube, corresponding to 2.5 nmol per liter of blood. Within- and between-batch CVs averaged 7.0 and 9.2%, respectively, over the working range of the assay (4.5–64 nmol/L). Concentrations of progesterone in blood spots (y) correlated well with those in serum (x) as measured by an established direct RIA (Clin Chem 28:1314, 1982): y = 0.430x – 2.44 (r = 0.972, n = 104). Progesterone is stable in the blood spots for at least 15 weeks at 25°C. The convenience of multiple sampling of blood by finger prick and the simplicity of the assay make this approach useful in investigating serial progesterone concentrations in outpatients.

Additional Keyphrases: capillary blood samples · steroid hormones · infertility evaluation · progesterone concentration during follicular and luteal phases · solid-phase double-antibody technique

The concentration of progesterone measured in serum in the mid-luteal phase of the menstrual cycle is widely used in the investigation of infertility, providing an index of luteal activity and presumptive evidence of ovulation. Recent simple RIA methods for progesterone in serum, which avoid the need for solvent extraction and involve an 125I tracer (1), are precise and robust, and discriminate well between follicular- and luteal-phase concentrations of progesterone. Despite these improvements in assay methodology, sample collection by venipuncture remains inconvenient and expensive, particularly when a series of blood samples is required. Progesterone measured in capillary blood, collected on filter paper after finger prick (2), or in saliva (3), could be determined in daily specimens collected at home, but such approaches require highly sensitive assays because of the small volume of blood assayed and the relatively low concentrations of progesterone in saliva.

RIA methods for blood-spot samples have been successfully used in measuring thyroxin and thyrotrpin in screening for hypothyroidism (4, 5) and 17-hydroxyprogesterone in detecting congenital adrenal hyperplasia (6, 7). The assay reported previously (2) for progesterone in blood spots had the advantage of convenient sampling but also had the practical disadvantage that it required extraction of a relatively large volume of blood, 50 μL, with solvent and use of a tritiated tracer.

Here we describe the development and evaluation of an RIA for progesterone in capillary blood, in which solvent extraction of the steroid is obviated and an 125I tracer is used with separation of bound and free fractions by use of a double-antibody solid phase. The working range of the optimized blood-spot assay is similar to that of the serum assays.

Materials and Methods

Reagents

All common reagents were of "Analar" grade, from BDH Chemicals Ltd., Liverpool, U.K. The synthetic androgen, danazol, was a gift from Winthrop Laboratories, Surbiton upon Thames, Surrey, U.K. Dextran T70 was from Pharmacia Ltd., Hounslow, U.K.

Buffer. Diluent assay buffer contained, per liter, 50 mmol of sodium phosphate (pH 7.4) and 2.5 g of bovine serum albumin ("RIA" grade; Sigma Chemical Co., Poole, U.K.).

Tracer. 125I-labeled progesterone-11α-glucuronyl-tyramine (125I-PGT) and Na125I were from Amersham International, Amersham, Bucks., U.K. (cat. nos. IM.140 and IMS.30).

Antiserum. The two antiserums to progesterone, raised in rabbits by injection with progesterone-11α-hemisuccinyl-bovine albumin, used in this study were a gift from Dr. J. E. T. Corrie. Antiserum raised in rabbit 30 had the highest avidity and was used for assay of progesterone in whole blood. The specificity of the antiserum has been reported, and a highly sensitive standard curve is obtained with 125I-PGT (8, 9). Antiserum raised in rabbit 31, of lower avidity, was used for assay of progesterone in serum (1).

Separation. "Sac-Cel," anti-rabbit IgG antibody bound to cellulose solid phase (cat. no. RD70), was from Wellcome Diagnostics, Dartford, U.K. Before use, we diluted it 10-fold in pH 7.4 buffer containing, per liter, 20 mmol of sodium phosphate, 10 mmol of EDTA (disodium salt), 50 g of Dextran T70, 145 mmol of sodium chloride, 500 mg of bovine albumin, 1 g of sodium azide, and 5 mL of Tween 20 (polyoxyethylene (20) sorbitan monolaurate).

Procedures

Preparation of standards. Method 1. We prepared blood-spot standards by using heparinized blood from men, to which we added an ethanolic solution of progesterone (Sigma Chemical Co.) to give a final concentration of 64 nmol per liter of blood (one volume of progesterone solution to 99 volumes of blood). This was further diluted serially to yield standards with assigned progesterone concentrations ranging from 0 to 32 nmol per liter of blood. We then spotted 25 μL of each blood standard onto filter-paper cards, let these dry horizontally at room temperature for 6–16 h, and stored them at room temperature in a sealed box in the presence of silica gel desiccant.

Method 2. We also incubated a filter-paper disc containing dried heparinized blood from men with 200 μL of progesterone standards in assay diluent containing 0, 30, 59, 119, 237, 475, 950, and 1900 pmol of progesterone per liter, corresponding to 0, 1, 2, 4, 8, 16, 32, and 64 nmol per liter of

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blood (based on 5.9 μL of blood per disc, see Results). These diluent standards in 500-μL aliquots were stable for as long as six months when stored at ~20 °C.

Blood spots for analytical-recovery studies were prepared in the same way, by adding progesterone to blood from men to give final concentrations of 7, 15, and 25 nmol per liter of blood.

**Blood-spot assay.** Duplicate discs of blood-spot standards (Method 1) and unknowns were placed in 10 × 75 mm glass assay tubes and eluted by exposure to 200 μL of assay diluent for 2 h at room temperature, without shaking.

For standardizing by Method 2 we added a disc containing blood from men to 200 μL of progesterone standards in assay diluent. After incubating the discs in diluent for 2 h at room temperature, we gently removed them from the assay tubes with a fine wooden stick. To the eluate we then added 50 μL of 125I-PGT (5000 counts/min, corresponding to about 5 fmol of PGT per tube) and 50 μL of progesterone antiserum (initial dilution 1800-fold) to the assay tubes. After incubating this mixture for 3 h at room temperature, we added 1 mL of the Sac-Cel suspension and incubated for a further 30 min at 4 °C, then centrifuged (2000 × g, 15 min, 4 °C). The supernates were decanted, and the radioactivity remaining in the pellets was counted in an LKB Multi-Gamma counter (LKB, Bromma, Sweden) for 2 min.

**Comparison method.** We also measured progesterone in serum by a direct RIA involving 125I-PGT and antiserum to progesterone 11α-hemisuccinyl-bovine albumin (1). The sensitivity of the assay was 2.5 nmol/L, the working range 4–100 nmol/L, the within-batch CV <8%.

**Calculation and analysis of results.** The radioactivity counts were processed on a Cipher micro-computer, with use of a five-parameter log-logit curve fit. We calculated precision profiles with an Apple II micro-computer, using the WHO Data-Processing Program developed at the Middlesex Hospital Medical School, London. For regression analysis the data were fitted to the straight line of least mean-squares best fit for the values of blood progesterone and the corresponding concentrations of detectable progesterone in serum.

**Samples**

Paired specimens (n = 179) of capillary blood, obtained by self-inflicted finger prick and collected onto filter paper, and samples of venous blood were obtained daily from six women volunteers throughout one menstrual cycle. The cycles were 24–33 days in length, and concentrations of estradiol and lutropin (>957 pmol/L and >42 int. units/L) and mid-luteal-phase progesterone (>42 nmol/L) in the sera were consistent with ovulation. Capillary blood was collected onto filter paper (Schleisser and Schuell Grade 2992, no. HMR 101/6; available from the Dept. of Health and Social Security, London, U.K.), from which we cut out discs 5.0 mm in diameter with a paper punch.

**Results**

**Analytical Variables**

**Determination of the volume of blood per disc and volume of eluate absorbed by the disc.** We estimated the volume of blood per disc by adding Na125I to whole blood from women (1.96 × 10^6 counts/min per milliliter). The mean radioactivity measured in 50 of the discs was 11 650 (SD 387) counts/min, corresponding to 5.9 μL of blood per disc or 3.4 μL of plasma, if a mean hematocrit of 42% in women is assumed. Mean radioactivity remaining in the eluates after removal of the discs was 10 350 (SD 340) counts/min, indicating that 22 μL (11%) of the eluate containing the analyte was absorbed by the disc.

**Elution of progesterone from filter paper.** We determined the time required for greatest elution of progesterone with diluent buffer from data on progesterone measured in discs spotted with blood for analytical recovery studies. These were eluted for intervals ranging from 0.5 to 12 h at 4 °C, and compared with blood-spot standards eluted for 16 h at 4 °C. Analytical recovery of progesterone was essentially quantitative and values were maximal within 2 h. To examine the relation of hematocrit to the elution and assay of progesterone, we prepared blood-spot standards, using blood from men, with the hematocrit adjusted to 38, 42, and 46%. These standards all gave closely similar standard curves; evidently hematocrit has little if any effect. Furthermore, no relationship could be demonstrated between the result for progesterone and the site of punching when the discs used as samples were punched from central and peripheral areas of blood spots of different sizes.

**Performance of progesterone assay.** The percentage of tracer bound in the zero standard in the presence of diluent alone, 49.3%, was decreased in the presence of eluates of blood spots from eight male volunteers (mean 40.8%, range 38.3–42.7%), indicating the need to compensate for this effect by including eluates of whole-blood samples in the standard curve. We selected blood from one of the volunteers, with 40.6% binding, for subsequent use in standardization. Figure 1 shows standard curves for progesterone in the presence of diluent buffer, compared with blood-spot standards (Method 1) and with diluent standards with the addition of a disc containing blood from a man (Method 2); it confirms that there is a diminution in specific binding and that binding characteristics are altered owing to effects of the eluate of blood from men. For comparison with the diluent standards, the concentrations of the blood standards (Methods 1 and 2) were decreased by 11% to correct for the loss of 22 μL of the eluate owing to removal of the discs before assay. The disc was removed before the RIA, because nonspecific binding increased from 2.9% to 11.3% when it was included, possibly owing to adsorption of the 125I-PGT tracer onto the filter paper.

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Fig. 1. Progesterone standard curve prepared with analyte in the presence of diluent alone (O) compared with standard curves prepared with use of blood-spot standards (C) (Method 1) and from diluent-based standards mixed with a disc of filter paper containing blood from a man (△) (Method 2). For comparison with diluent-based standards a correction factor of 11% has been applied for loss of eluate when filter paper discs are removed before assay (O and △). Nonspecific binding was respectively 2.1% and 2.3% in the presence of diluent alone (O) and in eluates of blood spots (C).

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We examined the need to include in the assay an agent to displace progesterone from binding proteins, as is done in direct assays for serum progesterone (1, 10). Danazol, 30 ng per assay tube, was included in the assay in similar proportions relative to the volume of serum to that used in direct serum assay. Standard curves for blood spots in the presence or absence of danazol were closely similar. Evidently danazol is superfluous.

The affinity constant for the antiserum was $1.4 \times 10^{10}$ L/mol, and the progesterone standard curve was highly sensitive. The limit of detection, defined as the lower 95% confidence limit of the zero standard, was 4.7 pg per tube, corresponding to 2.5 nmol of progesterone per liter of blood, if 5.9 µL of blood per disc is assumed. The working range of the standard curve is 4.5 to 64 nmol per liter of blood, corresponding to a CV of <12% based on the within-batch precision profile (Figure 2). The within-batch CV, based on eight determinations in duplicate on three recovery pools, averaged 7.0% (range 5.5–8.9%); the between-batch CV, based on 13 determinations on the same pools, averaged 9.2% (range 5.7–13.8%) (Table 1). Recovery of progesterone, based on 8 duplicate determinations, averaged 99% of the theoretical value in the dose range 7.0 to 25 nmol per liter of blood.

**Stability and storage of blood spots.** To determine the stability of progesterone in blood dried on filter paper under different conditions of storage, we stored blood spots of recovery pools (7, 15, and 25 nmol/L) either open to the atmosphere (4 °C and 25 °C), in the presence of high humidity (at 37 °C), or in the presence of silica-gel desiccant (at 4, 25, or 37 °C). We then assayed at intervals during 15 weeks, using freshly prepared blood-spot standards. Progesterone was found to be stable in blood spots stored at 4 or 25 °C (either open to the atmosphere, or in the presence of desiccant) for at least nine and 15 weeks, respectively (Figure 3). Progesterone was also stable at 37 °C in the presence of desiccant for at least nine weeks, although significant losses occurred within one week in the presence of high humidity.

**Comparison of Progesterone Concentrations in Serum and Blood**

Progesterone was undetectable in both serum and blood in 88 of 179 paired specimens, and progesterone was undetectable (<2.5 nmol/L) in 22 specimens of blood with corresponding concentrations in serum ranging from 3.4 to 12 nmol/L (median, 6.6 nmol/L). Progesterone was detectable in blood and serum in 82 paired specimens. Regression analysis gave the relationship $y$ (blood) = 0.430 ± 0.010 (SE)$r^2 = 2.44$ ($r = 0.972$, $n = 104$), based on all paired specimens with detectable concentrations in serum, including those in which progesterone was undetectable in whole blood. In such cases we included in the calculation concentrations in blood that were at or just below the formal limit of detection of the assay (Figure 4). For values of progesterone in serum exceeding 10 nmol/L, the variance of blood progesterone about the line of best fit was nearly independent of the value for serum progesterone. The standard error of a single determination of progesterone in serum from a single measurement of progesterone in blood was 5.6 nmol/L, and it was almost independent of the progesterone concentration in serum.

![Figure 2](image)

**Fig. 2.** Typical standard curve (■) and within-batch precision (○) of assay for progesterone in blood spots

The precision profile is the average of five consecutive assays, each consisting of at least 50 unknown determinations in duplicate.

<table>
<thead>
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<th>Added Progesterone, nmol/L</th>
<th>Recovered, nmol/L</th>
<th>Recovery, %</th>
<th>CV, %</th>
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In assessing within-assay precision, we assayed samples from pooled blood eight times, in duplicate. Recovery and between-assay data are results of 13 consecutive assays.

![Figure 3](image)

**Fig. 3.** Stability of progesterone in whole blood dried on filter paper stored at (a) 4, (b) 25, or (c) 37 °C

Blood spots from pooled blood containing 7 (■), 15 (○), or 25 (□) nmol of progesterone per filter were stored in the presence of desiccant (filled symbols) or open to the atmosphere (open symbols: a, b, or c) in high humidity (open symbols in c) for as long as 15 weeks until assayed and compared with freshly prepared blood-spot standards.
Figure 5 shows profiles of the concentrations of progesterone in blood and serum in the six volunteers, related to the lutein peak as day 0. Progesterone was uniformly undetectable in whole blood throughout the follicular phase of the cycle, and discrimination was good between concentrations in the follicular and mid-luteal phases. Progesterone concentrations in blood ranged from 6.2 to 31 nmol/L between five and nine days prior to menses; the corresponding concentrations in serum were in the range 23 to 77 nmol/L.

**Discussion**

The direct assay of progesterone in the eluent of 5.9 μL of blood required the development of a highly sensitive RIA, which we achieved by using a high-avidity antiserum raised to progesterone-11α-hemisuccinyl–bovine albumin, and 125I-PGT as radioligand. This combination of reagents, which is heterologous with respect to the chemical bridge in the immunogen and radioligand (8), yields a sensitive standard curve and has previously been used in a direct assay for progesterone in saliva (9). The detection limit, 4.7 pg/tube (corresponding to 2.5 nmol per liter of blood) represents at least a 10-fold increase in sensitivity as compared with most RIA methods for serum progesterone.

Eluates of blood from different men variably depressed specific binding and slightly changed the shape of the dose–response curve. Blood from men has a progesterone concentration of <0.5 nmol/L, and cross reactivity with other steroids is low (8), so we assumed that this reduction in binding reflects the effects of different blood matrices. We prepared standards in blood from a selected male donor in an attempt to equalize matrix effects in standards and unknowns. This approach has been widely used to directly assay various analytes in serum (1), saliva (9), and blood (4), and the close relationship found between progesterone concentrations in blood and serum suggests that use of blood from a selected man in preparing the standard curve is a practical and satisfactory method of compensating for matrix effects. Although we used blood-spot standards in developing the blood-progesterone assay, we subsequently used diluent-based standards with addition of a disc containing blood from men (Method 2) for routine assays of clinical specimens because it was simpler and required less blood.

We found progesterone in whole blood dried on filter paper to be stable at ambient temperature for as long as 15 weeks, which facilitates storage and transfer of blood spots to the laboratory.

A displacing agent such as danazol (10) is required in assays of serum, to displace progesterone from the proteins binding it in serum. Inclusion of danazol in the blood spot assay had an insignificant effect on the standard curve, suggesting that progesterone in the eluate is assayable without the need for a displacing agent. Possible reasons for this include the small sample volume, the high avidity of the antiserum, and denaturation of the binding proteins on filter paper.

We obtained the following direct relationship between progesterone in capillary blood (y) and serum (x); y = 0.430x - 2.44. The concentrations found in blood were about 43% of those in serum, rather than the expected 58% based on a mean hematocrit of 42% in women and assuming no binding of progesterone by erythrocytes (11). The disparity between measured and theoretical concentrations in blood remains, at least in part, unexplained. Standard curves obtained with blood-spot standards and diluent-based standards with the addition of a disc of blood from a male were similar, which suggested complete elution of progesterone from the filter paper. Also, analytical recovery of progesterone added to men’s blood averaged 93% of theoretical. Profiles of progesterone concentrations in blood and serum in the six subjects studied were closely similar, and the correlation coefficient was 0.98 for the relationship between concentrations in blood and serum. This suggests that the blood-spot assay is well suited for its major application in clinical practice, namely the measurement of progesterone in the mid-luteal phase of the cycle for assessment of corpus luteum function, to identify presumptive ovulatory cycles in which concentrations in serum are generally >25 nmol/L (12). Concentrations in both blood and serum were uniformly undetectable.
(<2.5 nmol/L) in paired specimens collected during the follicular phase. Progesterone was undetectable in 22 blood specimens collected during the early and late luteal phases when corresponding concentrations in serum ranged from 3.4 to 12 nmol/L, suggesting that measurements in blood are less satisfactory than those in serum for discriminating concentrations in the follicular phase from those in the early and late luteal phases of the cycle. Further studies are required to establish reliable reference ranges for blood progesterone and to assess the use of the assay in the investigation of infertility. Nevertheless, the convenience of the sampling technique and the simplicity of the assay allow assessment of luteal function to be based on a profile of concentrations in blood collected at home, rather than that in a single specimen collected on a selected day of the cycle.

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