THE MEASUREMENT OF OESTRADIOL AND PROGESTERONE IN PLASMA FROM NORMAL, INFERTILE AND CLOMIPHENE TREATED WOMEN

By

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ABSTRACT

The use of clomiphene to assess ovarian function in women has been monitored by the determination of plasma oestradiol and progesterone concentrations. The assay of both hormones was carried out on one plasma sample after separation of oestradiol and progesterone by TLC. The oestradiol was determined by double competitive binding to rabbit uterine supernatant (Robertson et al. 1971) and the progesterone by competitive binding to corticosteroid binding globulin. After clomiphene stimulation the oestradiol levels in the follicular and luteal phases exceeded those found in normal cycles; this was also true of progesterone concentrations during the luteal phase. The methods of assay were also used to follow changes in plasma hormone levels during the early stages of pregnancy in hitherto infertile women. These cases included a twin pregnancy and a pregnancy which terminated in abortion at 7 weeks.

Several methods are currently used to follow ovarian response to treatment designed to stimulate ovulation in women. These methods include variations in basal body temperature, examination of cervical mucus, endometrial and vaginal cytology and the serial determination of the urinary excretion of oestrogens and pregnanediol. These methods are of clinical value but in many cases they do not give a sufficiently clear cut result and it is clearly desirable to have a more direct and quantitative assessment of ovarian response based on plasma hormone levels. Oestradiol and progesterone were determined in one plasma sample after separation by TLC. The oestradiol was determined by double competitive binding (DCB) to rabbit uterine supernatant (Robertson et al. 1971) and the progesterone by competitive binding to corticosteroid
binding globulin (CBG). Plasma oestradiol and progesterone measurements before and after clomiphene treatment («Clomid», clomiphene citrate. Merrell Division, Richardson-Merrell Ltd., London) of a normal woman and several infertile women gave a clear indication of ovulation. This type of assay appears to have considerable value in detecting ovulation and in following the early stages of pregnancy.

**MATERIALS**

[2,4,6,7-³H] Oestradiol (100 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks., U. K. and was stored in benzene (20 ng/ml) at 6°C. After a short period of storage, approx. 2% of the total radioactivity migrated, in the TLC system used, with the same mobility as progesterone and as the specific radioactivity of the oestradiol was 2.5 times that of the [³H] progesterone used as internal standard, this impurity could introduce a serious error in the assessment of progesterone recovery. When necessary, the radioactive oestradiol was purified on a column of LH 20 Sephadex using the system benzene-methanol 85:15 (v/v) (Mikhail et al. 1970). After purification of the oestradiol, the error in the determination of progesterone recovery was less than 5%. Attempts to purify [³H] oestradiol by TLC on silica gel were not successful (cf. Coyntupa et al. 1970). [1,2-³H] Progesterone (41 Ci/mmole) and [1,2-³H] corticosterone (38 Ci/mmole) were obtained from the Radiochemical Centre, Amersham. The former was stored in benzene (10 ng/ml) at 6°C and was routinely checked for purity by TLC on silica gel in two systems (cyclohexane-ethylacetate 6:4 and chloroform-acetone 9:1 (v/v)). [³H] Corticosterone was stored in ethanol (40 ng/ml) at 4°C.

The buffer used for the determination of oestradiol was 0.01 m tris-HCl pH 8.0 containing 0.001 m EDTA and 0.25 m sucrose. That for the progesterone assay was 0.04 m sodium phosphate pH 7.2. The charcoal suspension used to remove free [³H] corticosterone contained 0.25% Norit A in phosphate buffer. The marker dye F9 (Lacquer violet, 1,4-diamino-anthraquinone) was supplied by The Imperial Chemical Industries Ltd., Dyestuff Division.

The radioactivity of samples (0.05–0.5 ml in buffer) was determined by transferring to counting vials containing toluene scintillation fluid (10 ml). The vials were agitated and allowed to stand in the dark for 2 h before counting in a Packard liquid scintillation spectrometer (Model 3375) (Mešter et al. 1970). The counting efficiency was approx. 45–50%.

**METHODS**

*The collection and extraction of blood samples*

Blood was collected in heparinized tubes and the plasma was stored at -10°C prior to processing. Without clomiphene treatment, blood samples were collected throughout the menstrual cycle 3 times during each week at the same time of day. Women treated with clomiphene («Clomid») received 50–200 mg/day for 5 days and the dosage was commenced, when possible, between the 2nd and 5th day of the cycle. Blood samples were collected at weekly intervals.
The plasma samples in duplicate (0.2–1.0 ml) were transferred to tubes containing internal standards, \( ^3 \text{H} \) oestradiol (50 pg; \( \sim 40 \,000 \) dpm) and \( ^3 \text{H} \) progesterone (25 pg; \( \sim 7000 \) dpm) and the contents were incubated at room temperature for 1–2 min. Peroxide-free ether (8 ml) was added and the tubes were shaken on a mechanical agitator (Whirlimix; Fisons Scientific Co. Ltd., Loughborough, Leics., U.K.) for 20 seconds. The contents of the tubes were centrifuged and the dried ether extracts were transferred to a series of parallel tracks, 1.5 cm wide made on a 20 × 20 cm silica gel plate (Merek Silica gel HR puriss purified by the method of Melider et al. (1971). The transfer was made in ether and a marker dye was also placed at the origin of each track. The dye (F9; Lacquer violet, 1,4-diaminoanthraquinone) had been resolved into a fast (F9\(_1\)) and a slow-moving (F9\(_2\)) component by column chromatography on Sephadex LH 20 using benzene-methanol, 8:15 (v/v) as the eluting solvent. The faster component (F9\(_1\)) which subsequently ran with the same mobility as progesterone was placed on all tracks receiving plasma extract. Both fast and slow components (F9\(_1\) and F9\(_2\)) were placed on each outer track and on a central track to ensure uniform running of the plate. These tracks contained no sample. The thin-layer plates were run without pre-equilibration in the solvent system chloroform-acetone 9:1 (v/v). The faster component (F9\(_1\)) which ran with the same mobility as progesterone (\( R_F = 0.53 \)) did not interfere with the assay of progesterone by competitive binding. Oestradiol (\( R_F = 0.25 \)) was separated on the thin-layer plates from both dyes (F9\(_1\), \( R_F = 0.53; \) F9\(_2\), \( R_F = 0.34 \)) (Table 1).

The zones of the tracks containing progesterone and oestradiol were located by reference to the coloured marker dyes and were collected by suction through Pasteur pipettes containing cotton wool plugs for progesterone and acid-washed glass wool

\[ \text{Table 1.} \]

\text{R}_F \text{ values of some steroids on silica gel thin-layer plates in the chloroform-acetone (9:1) solvent system.} \]

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_F )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>0.53</td>
</tr>
<tr>
<td>17-Hydroxyprogesterone</td>
<td>0.39</td>
</tr>
<tr>
<td>20( \alpha )-Hydroxy-pregn-4-en-3-one</td>
<td>0.31</td>
</tr>
<tr>
<td>20( \beta )-Hydroxy-pregn-4-en-3-one</td>
<td>0.34</td>
</tr>
<tr>
<td>Oestrone</td>
<td>0.47</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.25</td>
</tr>
<tr>
<td>Oestriol</td>
<td>0.06</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.25</td>
</tr>
<tr>
<td>Dihydrotestosterone (17( \beta )-hydroxy-5( \alpha )-androst-3-one)</td>
<td>0.38</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Marker dye F9(_1) fast migrating component (F9(_1))</td>
<td>0.53</td>
</tr>
<tr>
<td>Marker dye F9(_2) slow migrating component (F9(_2))</td>
<td>0.34</td>
</tr>
</tbody>
</table>

* The marker dye was a commercial sample of 1,4-diaminoanthraquinone.
plugs for oestradiol. The steroids were extracted with ether (~1 ml) and the extracts evaporated to dryness at 45°C. The recovery of both steroids was 40-50%.

**Assay of plasma oestradiol**

The measurement of plasma oestradiol was performed by the double competitive binding method (DCB) described in the preceding paper (Robertson et al. 1971). It was applied either directly to the plasma without TLC (direct method; DCB) or to ether extracts after TLC (indirect method; TLC & DCB).

**The effect of Clomiphene and its metabolites on oestradiol assay by CB**

Clomiphene is known to decrease the uptake of oestradiol by the rat uterus in vivo (Roy et al. 1964) and it seemed possible that this compound might interfere with the binding of oestradiol to uterine supernatant in vitro assay. Preliminary examination of the proprietary drug Clomid (clomiphene citrate + carrier material) indicated that in this form 4 ng of the preparation had the same effect on the binding of [3H] oestradiol as 0.1 mg of non-radioactive oestradiol.

When plasma samples were obtained during and after clomiphene treatment and these samples were assayed for oestradiol content by the direct (DCB) and indirect (TLC + DCB) methods, the latter method gave lower values during the period of administration of the drug. The difference between the two sets of values disappeared within 2-3 days of the cessation of treatment. An extract of plasma collected during clomiphene treatment at the beginning of a menstrual cycle was subjected to TLC on silica gel in the system cyclohexane-ethyl acetate 6:4 (v/v). The chromatogram track was subdivided at right angles to the direction of flow into a series of sections each of which was extracted and assayed by simple CB. Substances, other than endogenous oestradiol, were found which displaced bound radioactive oestradiol; one was eluted at the origin and the other had a mobility \( R_F = 0.31-0.56 \) (oestradiol \( R_F = 0.53 \)). These sources of interference were removed by the DCB (direct) method.

**Assay of plasma progesterone**

Progesterone was determined by competitive protein binding to corticosteroid-binding globulin (CBG) in the presence of [3H] corticosterone. The binding reagent was prepared from mongrel dog serum (10 ml) which was incubated with Norit A (50 mg) for 30 min at 30°C to dissociate and adsorb endogenous corticosteroids. The charcoal was removed by centrifugation at 4°C and the serum was stored in 0.5 ml portions at -10°C.

The optimum volume of serum for the assay of 1 ng of progesterone was determined by a preliminary experiment (c.f. Mešter et al. 1971). Increasing volumes of serum (0-10 µl) were transferred to a series of tubes containing 80 pg of [3H] corticosterone in 0.1 ml of phosphate buffer. A parallel series of tubes was prepared but in addition to the above components each tube contained 1 ng of progesterone. The volume in each was made up to 0.6 ml with buffer and the tubes were incubated for 10 min at 30°C and subsequently for a further 10 min at 0°C. Charcoal suspension (0.1 ml) was dispensed rapidly into each tube the complete operation being completed in 45 seconds. The tubes were shaken, stood for 5 min and centrifuged for 2 min at 4°C. A portion (0.5 ml) of each supernatant was counted for bound radioactivity.

The volume of serum which gave the largest change in percentage binding for 1 ng of progesterone was routinely used in the assay. Stock [3H] corticosterone-CBG solution was then prepared containing the optimum amount of [3H] corticosterone and
serum in 0.5 ml of phosphate buffer. This preparation was stored at 4°C for 2–3 days. Marked deterioration of the reagent was observed after 1 week.

The assay procedure

The residue containing progesterone obtained by ether extraction was dissolved in phosphate buffer (0.25–0.5 ml) by warming at 30°C for 2 min. A portion (0.1 ml) of this solution was set aside to assess recovery and a portion (0.1 ml in duplicate) was transferred to the assay tube. Progesterone was determined by reference to a calibration curve (0–2 ng) prepared simultaneously under identical conditions. [3H] Corticosterone-CBG preparation (0.5 ml) was added to all tubes which were incubated for 10 min at 30°C and subsequently for a further 10 min at 0°C. Charcoal suspension (0.1 ml) was added rapidly to each tube; the tubes were shaken and allowed to stand 3–5 min and were finally centrifuged at 800 g for 2–5 min at 4°C. A portion of the supernatant (0.5 ml) was counted for bound radioactivity. The amount of progesterone found in the extract by reference to the calibration curve (Fig. 1) and the percentage recovery of the internal standard were used to calculate the plasma concentration of the hormone.

Blank determinations were carried out by ether extraction of the progesterone region (F91) of tracks which received no plasma extract. These blank extracts represent traces of inhibiting compounds introduced from the thin layer plate and from solvents which were processed along with the assay samples. Negligible blank values were obtained with silica gel plates which were less than 2 days old. Significant blanks were obtained after 3 days. Close attention was necessary in the preparation, drying and storage of the plates.

The accuracy of the assay was determined by measuring amounts of progesterone (0.5, 1.0 and 10 ng) added to buffer and to steroid-free plasma (Table 2). Precision was calculated from the difference between duplicate determinations according to the formula of Snedecor (1952). In the range 1–10 ng progesterone per ml the method

![Calibration graph of progesterone (0-2 ng). Determinations were made by competitive binding to CBG (dog serum) in the presence of [3H] corticosterone. Each point is the mean of 5 determinations ± 1 sd. Details are given in the Methods section.](image_url)

Fig. 1.
Table 2.
Determination of standard amounts of progesterone added to buffer or steroid-free plasma.

<table>
<thead>
<tr>
<th>Progesterone added</th>
<th>Progesterone measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>0.5</td>
<td>0.68 ± 0.12 (n = 6)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.97 ± 0.05 (n = 5)</td>
</tr>
<tr>
<td>10.0</td>
<td>10.0 ± 1.2 (n = 5)</td>
</tr>
</tbody>
</table>

had a sd ± 0.84 ng/ml (n = 20) (sample size 0.5–1.0 ml) and in the range 10–100 ng per ml sd ± 1.5 ng/ml (sample size 0.1–0.5 ml).

[3H] Progesterone as internal standard
In principle the assay depends on the displacement of [3H] corticosterone by the non-radioactive progesterone present in the sample extract and the use of relatively large amounts of [3H] progesterone as internal standard may affect this equilibrium. The use of 25 pg of [3H] progesterone (7000 dpm) in the assay procedure was based on a preliminary study of the effect of increasing amounts of internal standard on the percentage binding of [3H] steroid in the presence of a fixed amount of non-radioactive progesterone. Portions (0.5 ml) of [3H] corticosterone-CBG reagent were incubated under assay conditions with 100 pg of non-radioactive progesterone and increasing amounts (0–10.7 pg) of [3H] progesterone. It is clear from the results of this experiment (Table 3) that up to 4 pg [3H] progesterone (~ 100 dpm) may be present under normal assay conditions without marked effect on percentage binding. This would correspond to the addition of 25 pg of radioactive progesterone to a

Table 3.
Influence of increasing amounts of [3H] progesterone added as internal standard on the percentage of [3H]-labelled steroid bound by CBG in the presence of 100 pg of progesterone.

<table>
<thead>
<tr>
<th>[3H] Progesterone added as internal standard</th>
<th>% of [3H]-steroid bound to CBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPM</td>
<td>pg</td>
</tr>
<tr>
<td>750</td>
<td>2.6</td>
</tr>
<tr>
<td>1250</td>
<td>4.4</td>
</tr>
<tr>
<td>2500</td>
<td>8.7</td>
</tr>
<tr>
<td>3125</td>
<td>10.7</td>
</tr>
</tbody>
</table>
plasma sample containing 500 pg of non-radioactive progesterone with procedural losses of 50–60%.

**Charcoal as an adsorbent of free [³H] steroids**

Conditions were found under which it was possible to use charcoal to adsorb free [³H] corticosterone without significant adsorption or dissociation of the [³H] corticosterone-CBG complex. By using Norit A at < 1 mg per ml of reaction mixture it was possible to limit the rate of dissociation or loss of the complex to 1–1.5% per min. By dispensing the charcoal suspension rapidly by an automatic pipette into all tubes within 40 seconds the difference between the [³H] corticosteroid bound before and after charcoal addition (40 seconds) was negligible. The tubes were promptly shaken to ensure complete mixing and they were left for 3–5 min at 0°C before centrifuging at 800 g, for 5 min at 0°C.

**RESULTS AND DISCUSSION**

The method was applied to measure oestradiol and progesterone concentration in plasma samples taken from a normal woman (40 a) at intervals throughout a 28-day menstrual cycle and the study was repeated with the same subject after the administration of clomiphene (100 mg for 5 days) at the beginning of a subsequent cycle (26 days). In both the normal cycle and that induced by clomiphene treatment, there was an increase in the plasma levels of oestradiol in both the follicular and luteal phases (Fig. 2). After clomiphene treatment, the oestradiol concentration in both phases was greater than that recorded in the normal cycle.

![Plasma oestradiol and progesterone concentrations](image)

**Fig. 2.**
Plasma oestradiol (○ --- ○) and progesterone (● --- ●) concentrations throughout the menstrual cycle of a normal premenopausal woman (40 years); --- normal cycle, --- after treatment with Clomiphene (100 mg for 5 days). The cycles are related to the first day of the menstrual cycle.
In the luteal phases of both cycles, normal and clomiphene induced, a rise in plasma progesterone concentrations reached a maximum at mid-phase and in this subject clomiphene produced higher values than those of the preceding normal cycle.

Fig. 3 illustrates changes in plasma hormone levels in normal women (9) and in women (10) with a history of infertility treated with clomiphene to induce ovulation. As many subjects in the latter group did not have regular periods, the cycles for comparison have been related to the first days of the subsequent menstrual period. The normal subjects (18–40 years) had cycles ranging from 20–35 days characterized, in most cases, by a biphasic pattern of increase in plasma oestradiol levels; during the follicular phase concentrations ranged from 20–50 ng oestradiol/100 ml and in the luteal phase from 10–30 ng/100 ml. Similar values have been reported by others (Korenman et al. 1970; Corker et al. 1970). After ovulation plasma progesterone concentrations also increased to maximum values during the luteal phase ranging from 10–20 ng progesterone/1 ml (c. f. Neill et al. 1967; de Souza et al. 1970; Martin et al. 1970). Not all women treated with clomiphene for infertility ovulated but those who had increased plasma progesterone concentrations during the luteal phase also gave enhanced oestradiol levels in the follicular and luteal phases. In the majority of such cases the increase in oestradiol concentration in both follicular and luteal phases was substantially above normal levels and on occasion reached 10-times the normal level. In a similar manner the increased plasma progeste-
rone levels after clomiphene response were substantially higher than levels encountered during the luteal phase of a normal cycle (Fig. 3).

A number of women treated with clomiphene to stimulate ovulation, conceived and plasma samples were obtained for assay during the first 8 weeks of pregnancy. The group examined included 7 pregnancies judged to be following a normal pattern at 16–20 weeks, 1 case of twin pregnancy. 1 case associated with very low plasma oestradiol levels and a pregnancy which terminated in abortion at 7 weeks. All time measurements were made from the first day of clomiphene treatment. In apparently normal pregnancies the plasma concentration of oestradiol during the first 5 weeks was of the same order as that encountered during the luteal phase of clomiphene treated women and thereafter began to increase (1–4, Fig. 4). Consistent increases in oestradiol concentrations were observed from week 2 onwards. In these pregnancies the plasma progesterone levels did not fall as in a menstrual cycle but remained at the level found during the luteal phase after clomiphene stimulation; with the exception of the twin pregnancy the levels did not rise notably even after week 5 (c. f. oestradiol). Subject 5 had very low plasma oestradiol levels even as late as week 8 and showed signs of bleeding at week 5; plasma progesterone levels were apparently normal and the subject was still pregnant at 22 weeks. The pregnancy which terminated in abortion at 7 weeks (6, Figs. 4 and 5) had given a good response to clomiphene treatment with raised plasma oestradiol and progesterone concentrations during the follicular and luteal cycles but these were not maintained. Falling oestradiol levels were recorded at week 3 and falling progesterone levels at week 5.

The increase in plasma progesterone levels in the luteal phase after clomiphene treatment suggests either an increase in steroid production of the corpus luteum, or more likely, the presence of more than one corpus luteum. It is unlikely that clomiphene would act directly on the corpus luteum as the circulating levels of the drug at this stage would be negligible (Kistner 1968).

The increase in plasma oestradiol levels after treatment with clomiphene is consistent with reports of elevated urinary oestrogens (Osmond-Clarke et al. 1968).

The decline in plasma oestradiol and progesterone concentrations in the case of abortion after six weeks gestation was associated with the failure of the corpus luteum rather than the placenta which has not, at this stage, taken over the endocrine function. According to Dignam (1968) abortions after 12 weeks are not usually associated with a decline in plasma progesterone levels.

The determination of plasma oestradiol and progesterone levels is not only of value in establishing that ovulation has taken place, but may also be used to confirm the onset and early stages of pregnancy. These measurements may also have value when applied to the study of women with a history of abortion in the early stages of pregnancy.
The concentrations of oestradiol (o—o) and progesterone (●—●) in plasma during the first 8 weeks of pregnancy after Clomiphene-induced ovulation. The pregnancies were dated from the first day of clomiphene treatment. Cases 1–4, apparently normal pregnancies; Case 3, twin pregnancy (twins born); Case 5, low initial oestradiol levels (bleeding at 5th week); Case 6, aborted at 7th week.
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