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RADIOIMMUNOASSAY OF ESTRONE* AND ESTRADIOL-17β IN PERIPHERAL PLASMA OF PREGNANT AND NON-PREGNANT WOMEN

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SUMMARY

Estrone and estradiol-17β have been measured in non-pregnancy plasma, by radioimmunoassay, after chromatographic separation on short Sephadex LH_{20} columns. Dextran coated charcoal has been used for adsorbing unbound estrogen in the incubation mixture. No meaningful results could be obtained by measurement of total free estrogens in crude extracts of non-pregnancy plasma.

In non-pregnancy plasma, the limited specificity of the antibodies imposed the chromatography step. The precision of the measurements during the menstrual cycle was of 9–15% (coefficient of variation).

In pregnancy plasma the direct measurement of free estrogens in crude extracts, without chromatography, gave results that were proportional to the volume of plasma extracted.

The precision of the measurements varied between 7–15% (coefficient of variation), for both low and high plasma concentration. In pregnant subjects a good correlation was found between the level of unconjugated estrogens in the plasma and the level of urinary estriol.

INTRODUCTION

THE DIRECT measurement of unconjugated estrogen level in the plasma, using double isotope dilution techniques[1, 2] or gas-chromatography, has been reported by several authors[3–6]. More recently, the competitive protein binding method[7–11] and the radioimmunoassay[12–14] permitted more accurate measurement of the low levels encountered in course of the menstrual cycle [15, 16]. It made possible the rapid determination of free estrogen in plasma during pregnancy[16, 17].

In the present work, we have analyzed the precision obtained with the radioimmunoassay for estrone and estradiol-17β measurements during the menstrual cycle and in the course of pregnancy.

MATERIALS AND METHODS

Antiestradiol antibodies were kindly supplied by Dr. R. Vande Wiele (Antibody A) and Dr. I. H. Thorneycroft (Antibody B). Both preparations were obtained after immunization of sheep with 17β-estradiol-17-hemisuccinate-bovine serum albumin. Antibody B had a higher tiler than antibody A. Antibody A was treated with Rivanol (2-ethoxy-6,9-diaminoacridine lactate) in order to remove most of the serum proteins except the gamma globulins[12, 18]. Both antibodies were diluted with 0·2 M phosphate buffer, pH 7·4, containing 1 g/l of

*Estrone, estradiol-17β and estriol will be referred to by E_1, E_2 and E_3 respectively.

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gelatine and 1 g/l of sodium azide to 1/10·000 or 1/20·000 for antibody A and to 1/25·000 for antibody B. Diluted antibodies were preserved at 4°C.

[6, 7-^{3}H]-estradiol-17β (NEN-50 Ci/mmol) was used. Radiochemical purity (more than 97 per cent) was ascertained by chromatography on Sephadex LH_{20} column as described below.

Column chromatography was performed on Sephadex LH_{20}, Pharmacia Fine Chemicals. The method was adopted from Mikhail *et al.* [13] as follows. Sephadex LH_{20} was suspended in benzene–methanol 93/7 and allowed to stand for 15 min. The floating fine particles were removed and excess benzene-methanol was added. After reposing overnight the supernatant was discarded and the Sephadex slurry was packed in small columns (i. d., 0·6 cm, height of packed sephadex, 7 cm). After benzene–methanol washing for 1 h the column was ready for use.

Charcoal suspension was prepared as follows: Norit A was suspended in distilled water and centrifuged 10 min at 1100 *g*. The supernatant was discarded and the procedure was repeated twice. The Norit A was then dried at 60°C for three days. 250 mg of dried Norit A and 25 mg of Dextran T_{70} (Pharmacia) were suspended in 100 ml of 0·2 M phosphate buffer containing 1 g/l of sodium azide and 1 g/l of gelatine.

Radioactivity was counted in a Tri-Carb Packard spectrometer, using the following scintillator mixture: dioxane (750 ml), ethylglycol (175 ml), naphtalene (45 g), DPO (9 g) and POPOP (450 mg).

All solvents were analytical grade and used without further purification.

METHOD

Blood was drawn in heparinized tubes and plasma separated after 10 min centrifugation at 700 *g*, at room temperature. Control assays using [6,7-^{3}H]-estrone and [6,7-^{3}H]-estradiol as tracers, have shown that under these conditions, no significant interconversion occurred between E_1 and E_2 *in vitro*.

Plasma was kept frozen until the extraction was performed.

1 ml of plasma was extracted twice with 5 ml of diethylether. The ether was removed under nitrogen and the extract was diluted in ethanol when a direct assay was to be performed. Otherwise, it was dissolved in 0·1 ml of benzene–methanol (93/7) and deposed on top of the Sephadex LH_{20} column.

Sephadex LH_{20} column prepared as described above, was eluted with increasing volumes of benzene–methanol (93/7) in order to isolate estrone (eluate II) and estradiol (eluate III). If desired, estriol could also be easily obtained (eluate V) (Fig. 1).

The overall recovery was about 90% and no correction was found necessary.

Incubation procedure and separation of bound estradiol were performed as suggested by I. H. Thorneycroft [19].

Appropriate aliquots were taken in duplicate, either from the ethanol dilution of the ether extract or from the eluates corresponding to E_1 and E_2, so that the assay tubes contained between 20–400 pg.

Aliquots were evaporated and 0·1 ml of buffer containing approximately 5000 cpm of [6,7-^{3}H]-E_2 ($\simeq$ 30 pg), was added to each assay tube. 0·1 ml of the antibody solution was then added, and allowed to incubate at 4°C for 16 h. 1 ml of cold charcoal solution was then added and the assay tubes were shaken for 20 sec on a Vortex mixer. The charcoal was precipitated by centrifugation at 1100*g* for

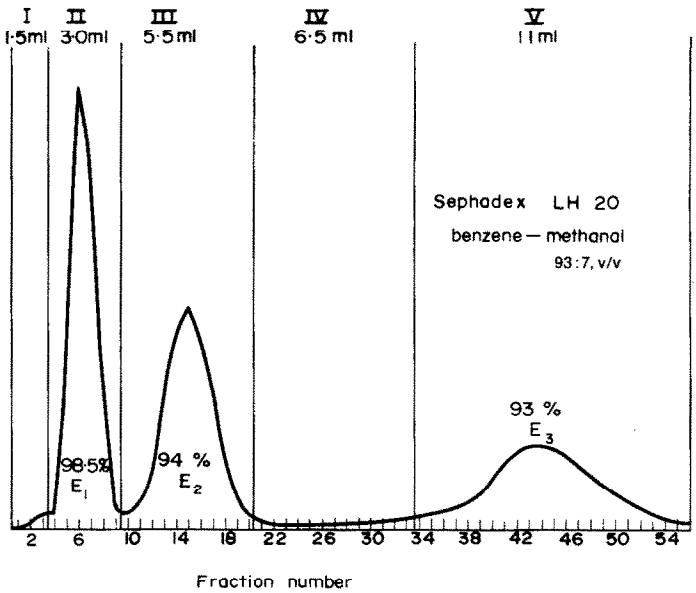

Fig. 1. Chromatography of estrone (E_1), estradiol-17β (E_2) and estriol (E_3) standards on a small column of Sephadex LH_{20}. Elution solvent: Benzene-methanol 93 : 7, v/v. Elution volumes, as well as % recovery of each standard are indicated.

15 min, at 4°C. 0·8 ml of the supernatant were transferred to counting vials.

Standards in duplicates, ranging from 16 to 1·000 pg were run simultaneously along with blanks (residues of 15 ml aliquots of diethylether evaporated to dryness). The latter were always negligeable and no correction was made for blank values.

Controls containing the radioactive tracer only were taken as the 100% reference binding (see Figs. 2 and 3). The effective binding in these controls varied from 65 to 90%, depending on the antibody dilution.

ANALYSIS OF THE METHOD

(1) *Standard curves*

Figure 2 gives typical standard curves obtained with antibody A, at 1/10·000 and 1/20·000 dilutions.

Means of triplicates and SE are indicated.

Figure 3 gives the binding curves with antibodies A (1/10·000) and B (1/25·000) for E_1, E_2 and E_3, using [6,7-^{3}H]-estradiol as tracer. Cross reactions were approximately the same for both antibodies: 30–50% for E_1 and 3–5% for E_3. It should be noted however that the binding curves were not parallel. Hence, interference was more important at lower than at higher concentrations.

(2) *Plasma extracts*

(a) Direct assay of unconjugated estrogens

(1) *Relationship between volume of plasma and estrogen concentration outside pregnancy.* Six measurements were done on extracts of 0·1, 0·2, 0·4, 1·0 or 2·0 ml of the same plasma pool, having low estrogen levels.

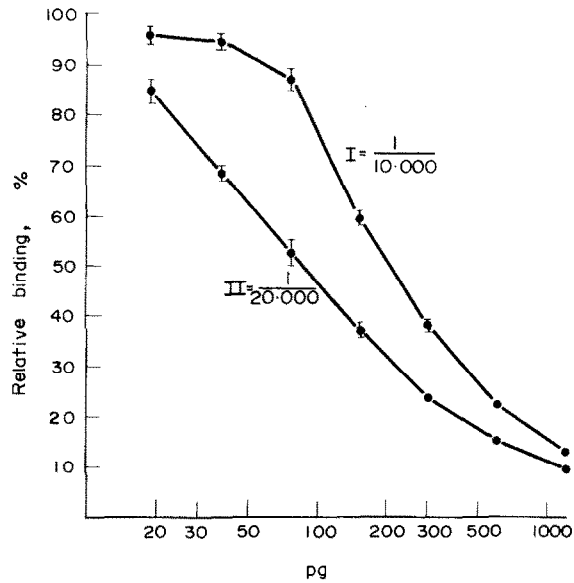

Fig. 2. Typical standard curves for estradiol-17β at two dilutions of antibody A.

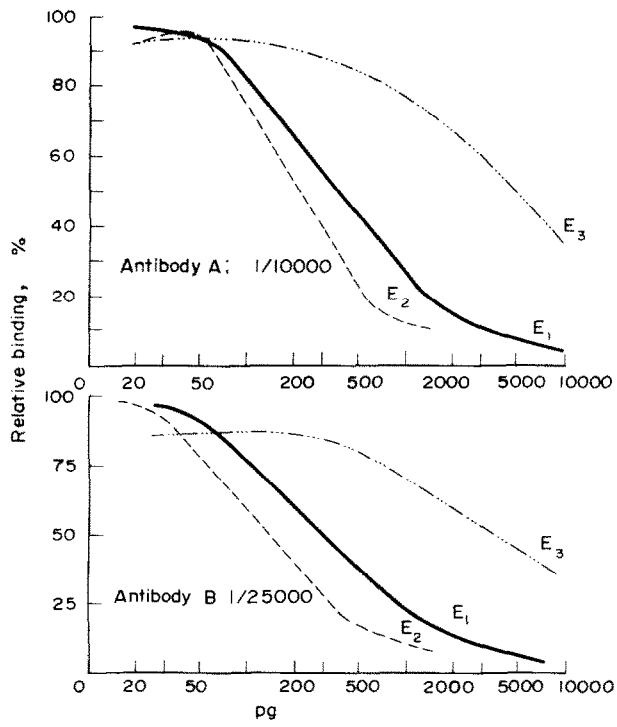

Fig. 3. Cross-reactions of E_1, E_2 and E_3 with antibody A (upper part) and antibody B (lower part).

The coefficient of variation of the percentage relative binding varied between 4 and 6 per cent for each of the 5 groups. However, the concentration of estrogen per ml of plasma fell from 462 to 44 if the plasma sample was increased from 0·1 to 2·0 ml. Hence, a correct value could not be obtained by direct assay in this case.

(2) *Recovery of added estradiol.* 17β-estradiol was added in increasing amounts (from 20 to 500 pg/ml) to 2 × 3 ml fractions of a plasma pool having a basal estrogen concentration of 94 pg/ml. All measurements were made in triplicates.

Table 1 shows that the increase of measured estrogens in the consecutive pool fractions compared to the basal concentration, corresponded quantitatively to the amount of added estradiol.

(3) *Precision of measurements in pregnancy plasma.* Table 2 gives the precision of estrogen measurements from duplicate determinations in pregnancy plasma samples. The volume of plasma varied from 2·5 to 200 μl, depending on the estrogen concentration. The coefficient of variation varied from 6·5 to 16% when duplicates of the same volume were performed. There was no difference in the results obtained from small or larger volumes of plasma. The precision did not vary at low (0·4–5 ng/ml) and high (20–40 ng/ml) plasma concentration.

Table 1. Recovery of added estradiol-17β to 3 ml portions of a plasma pool. Mean of six measurements. (Antibody A, 1/10·000)

Added E_2 (pg/ml)	Measured estrogens (pg/ml) Mean (extremes)*	Recovered estradiol (measured minus basal)
0 (basal level)	94 (88–101)	—
20	110 (102–121)	24
50	130 (106–158)	36
100	180 (166–200)	86
200	288 (266–322)	194
300	389 (367–411)	295
500	522 (483–556)	428

*Regression line: measured = 96 + 0·9 added.

Table 2. Precision of plasma unconjugated estrogen assay during pregnancy (duplicates), for different plasma volumes. (Antibody B, 1/25·000)

Range of Plasma levels (ng/ml)	N(1)	Plasma volume (μl)	Mean concentration ($\bar{x}$) (ng/ml)	$\bar{d}$(2) (ng/ml)	s(3)	$s/\bar{x}$ (%)
0·4–5	8	40–60	1·89	0·20	0·20	10·6
		100–200	1·55	0·13	0·10	6·5
5–10	9	10–30	7·51	1·10	1·20	16·0
		30–50	7·28	0·84	0·85	11·7
10–20	15	2·5–10	15·1	1·54	1·30	8·5
		12·5–30	16·0	1·53	1·44	9·0
20–40	18	2·5–7·5	26·7	2·70	2·40	8·9
		12·5–25	27·1	2·13	1·84	6·8

(1) Number of duplicates; (2) mean difference between duplicates. (3) Standard deviation estimated from the difference in the duplicates: $s = \sqrt{\Sigma d^2/2N}$.

(b) Estrone and estradiol measurements after column chromatography

(1) *Relationship between plasma volume and estradiol concentration.* After chromatographic isolation from different volumes of the same plasma pool, unconjugated estradiol concentration was independent of the volume of the plasma sample (Table 3).

(2) *Comparison of estradiol-17β concentration in pregnancy plasma by direct assay and after chromatographic isolation.* Table 4 shows that, for 4 pregnant plasma samples, the mean plasma concentration of estradiol-17β was

Table 3. Relationship between plasma volume and estradiol assay in non-pregnancy plasma pools, after Sephadex chromatography (Antibody A, 1/20·000)

Plasma volume (ml)		Measured E_2 (pg)
Pool 1	2	218 (189; 246)
	4	561 (539; 583)
Pool 2	5	132 (144; 120)
	10	267 (267; 267)

Table 4. Levels of unconjugated estrogens (1) and of estradiol-17β isolated by Sephadex chromatography (2) in 4 pregnancy plasma samples. (Antibody B, 1/25·000)

		Mean ± s (ng/ml)	
Plasma	n	1 (No chromatography)	2 (Sephadex chromatography)
S	6	16·0 ± 1·2	16·4 ± 1·2
R	6	19·7 ± 1·2	18·3 ± 1·8
M	6	13·5 ± 0·8	12·4 ± 0·4
VG	6	19·2 ± 1·8	17·1 ± 1·6

Table 5. Precision of plasma assay of estrone and estradiol-17β during the menstrual cycle (Duplicate*). (Antibody A, 1/20·000)

Amounts in assay tube (pg)	N	$\bar{x}$ (pg)	$\bar{d}$ (pg)	s	$s/\bar{x}$ (%)
Estrone					
20–50	33	42·8	6·8	5·9	13·8
50–100	59	75·2	13·0	12·0	16·0
100–250	26	148	21·8	21·3	14·4
Estradiol					
20–50	41	36·6	5·7	5·0	13·6
50–100	43	71·9	7·5	7·6	10·6
100–250	41	181	15·4	15·8	8·8

*Legends, see Table 2.

slightly lower after chromatographic separation, as compared to the direct assay of estrogens.

(3) *Precision of estrone and estradiol-17β measurements during the menstrual cycle.* Table 5 gives, for different ranges of hormone levels in the assay tubes, the precision of duplicate measurements of estrone and estradiol-17β. The coefficients of variation were lower for estradiol-17β (8·8–13·6%) than for estrone (13·8–16%).

RESULTS

(1) *Estrone and estradiol-17β concentration in the plasma during the menstrual cycle*

Figure 4 shows the plasma concentration of E_1 and E_2 during the menstrual cycle of a normal woman. As generally described by others [13, 15, 16] two peaks were also observed; the first one preceeded the LH* peak; the second coincided with the progesterone* peak. A more extensive study of the normal cycle will appear elsewhere.

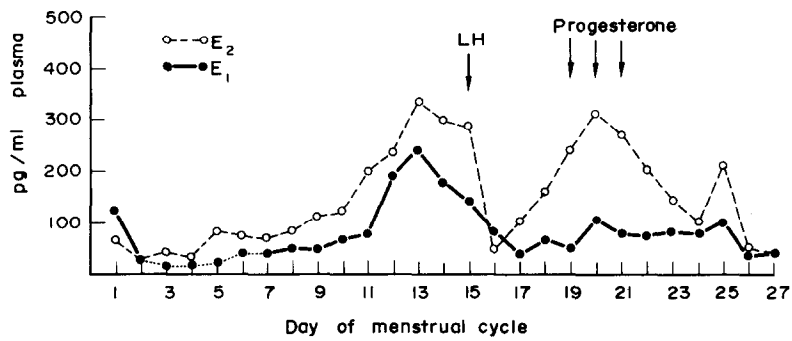

Fig. 4. Plasma levels of E_1 and E_2 during one menstrual cycle.

(2) *Estrogen concentration in pregnancy*

Plasma estrogens measured by the direct assay, were determined in 81 samples, at different stages of pregnancy. Table 6 gives the results obtained and their standard deviations. Figure 5 shows the correlation between estrogen concentration in plasma and estriol measurement in the urine (determined by fluorimetry) in 58 samples. The coefficient of correlation (0·80) was highly significant ($p < 0{\cdot}001$).

DISCUSSION

The radioimmunoassay of unconjugated estrogens in blood has opened new possibilities in clinical research. However, meaningful results will be obtained only if the specificity of the measurement is adequate. Specificity of the antisera to 17β-estradiol-17-hemisuccinate-bovine serum albumin used in the present work has been extensively studied by Mikhail *et al.* [13] and by Thorneycroft *et al.* [14]. The 30–50% cross reaction with estrone and 3–5% with estriol reported here match with their findings.

*Plasma samples were obtained through the courtesy of Dr. K. Thomas, University of Louvain, who also performed the measurements of LH. Progesterone levels were measured by Dr. M. Pizarro, of Louvain.

Table 6. Levels of plasma unconjugated estrogens during pregnancy

Weeks	n	Mean $\pm s$ (ng/ml)
6–10	6	0·9 ± 0·6
11–15	6	3·5 ± 2·6
16–20	10	7·1 ± 3·9
21–25	5	11·0 ± 2·9
26–30	11	20·0 ± 8·6
31–32	12	23·3 ± 9·7
33–34	8	23·5 ± 9·0
35–36	14	23·4 ± 6·3
37–40	9	28·7 ± 10·9

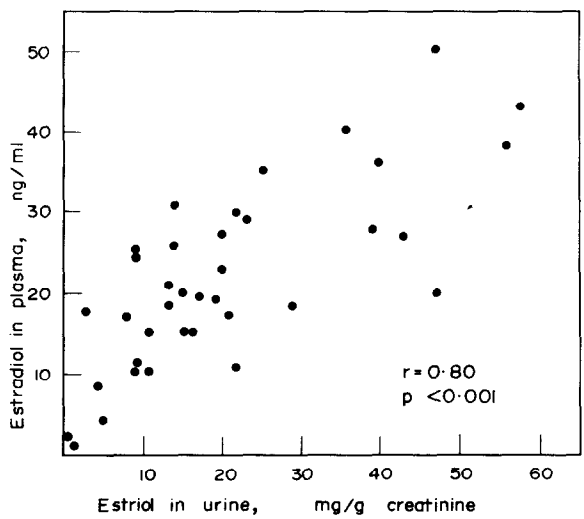

Fig. 5. Correlation between plasma unconjugated estradiol and urinary estriol in pregnancy.

Cross reaction with C_{19} steroids, progesterone and corticosteroids is negligeable[13, 14], and can be disregarded in female plasma extracts.

Practically, the specificity of the radioimmunoassay would never be ascertained in unpurified plasma extracts, as unknown compounds may interfere with the reaction. One necessary, although not necessarily sufficient, condition to be met before specificity can be considered is that the result of the assay be independent of the volume of the extracted plasma.

In our study, this condition was met in non-pregnancy plasma, provided estrone and estradiol-17β were isolated on small columns of sephadex LH_{20}. If not, the results were dependent on the volume of plasma extracted; the apparent estrogen concentration was inversely related to that volume.

This observation depended on the volume of plasma itself, and not on the amount of estrogen, since increasing amounts of estradiol-17β added to the same volume of plasma gave quantitative recoveries at all hormone concentrations from

20 to 500 pg/ml. The lack of proportionality between different plasma volumes may be due to the presence in the plasma of some cross-reacting material, possessing reaction kinetics different from that of estradiol. It should be noted here that the cross-reaction with estrone and estriol (see Fig. 3) yielded standard curves which did not parallel the estradiol standard curve. Therefore, interference of these hormones with estradiol will be comparatively more important at lower than at higher concentrations. It is however unlikely that the lack of proportionality observed in this study is due only to these compounds, usually present in too low concentrations in the plasma[20, 21] (see also Fig. 4). Small amounts of other unconjugated estrogens have been described in the plasma of non-pregnant[22] and pregnant[23] women, and might possibly cross-react with the antibodies. Precise data however are lacking in that respect. Other substances, even of a non steroid nature, might possibly also interfere and prevent meaningful results to be obtained for any plasma volume.

The chromatography of plasma extract on small Sephadex LH_{20} column was quantitative and reproducible (see Fig. 1). By careful calibration of the column, the elution volumes were precise and the incorporation of a marker as proposed by Castanier and Scholler[24] and also used by Emment *et al.*[20] was not necessary. Also, the whole technique involved a loss of less than 10–15%. Hence, incorporation of a tracer to the plasma for recovery measurement which would complicate later the radioimmunoassay procedure was unnecessary.

The precision of the method was of the order of 10–15% (coefficients of variation) from duplicate measurements. The sensitivity has not been studied thoroughly. It depends on the affinity of the antibody, but also on the specific activity of the labeled tracer. It is indeed necessary to have enough counts in the bound form throughout the range of the standard curve, in order to avoid counting errors.

Using about 30 pg of [6,7-^{3}H]-estradiol (50 Ci/mmol) as the tracer, we found that the optimal dilutions were 1/20·000 for antibody A and 1/25·000 for antibody B. Under these conditions the smallest amount of estrogens required in the assay tube was about 20 pg. Consequently, estrone and estradiol-17β could be accurately measured in duplicates in 0·2 (peak levels) to 1·0 ml (basal levels) of plasma during the menstrual cycle (see Fig. 4).

In pregnancy plasma, the relatively high concentration of estradiol allowed to obtain meaningful results without chromatography, since proportionality between plasma volume and estrogen measurement was respected. On the other hand, the direct assay of total unconjugated estrogens and the measurement of estradiol isolated by Sephadex chromatography yielded rather similar results (see Table 4). This might be explained by the presence of relatively lower levels of unconjugated estrone and estriol during pregnancy[23, 25] and by the lower affinity of estrone for the antibodies (Fig. 3). Direct assay of estrogen offers the possibility of tracing the hormonal state during pregnancy by rapid results obtained from small volumes of plasma (2·5–200 μl). The precision was of the order of 10 per cent (coefficient of variation) for duplicates.

A good correlation was found between urinary estriol and plasma estrogen during pregnancy. This finding is in accordance with the resport by Tulchinsky *et al.*[25] who found a good correlation between plasma unconjugated estriol and estradiol. Plasma estradiol has also given a good correlation with clinical features of abnormal pregnancies[17, 26]. However, it should be remembered that

estradiol and estriol have slightly different origins: E_3 comes mostly from foetal sources, whereas a significant part of E_2 is of maternal origin. Hence, the clinical meaning of these two parameters might be slightly different, although data on this point are still lacking. On the other hand, the increase in plasma estrogens was measurable with high precision already from the first few weeks (see Table 6); it may then be more useful than urinary estriol in checking the hormonal status during the first half of pregnancy.

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