NOTE

Evaluation of a Rapid Competitive Protein Binding Assay for Progesterone in Pregnancy and some of its Complications

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Synopsis

The assay of serum progesterone using the competitive protein binding procedure allows rapid and precise measurements of progesterone and makes large population studies on various complications of pregnancy more feasible. Several important factors were found to influence the accuracy and reproducibility of the assay. The levels of serum progesterone in some normal pregnancy, hydatidiform mole and choriocarcinoma are presented.

Several publications have described the assay of serum and plasma progesterone (Szarka, 1940; Short, 1958; Greig *et al.*, 1962; Neil *et al.*, 1967; Weist, 1967; Yannone, 1968; Johansson, 1969; Rubin *et al.*, 1970; Murphy, 1971). Clinical and research applications for plasma progesterone determinations have been suggested (Johansson, 1969). However, the methods described involved slow and laborious techniques. It was desirable for us to investigate a method involving competitive protein binding (CPB) for progesterone assay which would be simple and rapid. Thus, the CPB method of Neill *et al.* (1967) was analysed to study the important factors that would influence the accurate determination of progesterone and also simplify the procedure to enable rapid assay of large number of samples.

Materials and Methods

Reagents

All reagents were used without further purification.

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tion. Florisil (Sigma Chemical Co., St. Louis, U.S.A.) was washed four times with water and activated by heating at 130°C. Activated Florisil was usable for at least two months.

Plasma from female volunteers after 14 days treatment with ethinyl oestradiol (50 µg/day) was used as the source of corticosterone binding globulin (CBG).

Extraction and Separation

Pregnancy serum (0.1 ml) was extracted with 10 volumes (1 ml) of petroleum ether. The extract was eluted on a 25 × 1 cm Sephadex LH-20 column using the chloroform-heptane-ethanol mixture of Murphy (200:200:1, v/v/v) (Murphy, 1971). Progesterone is found in the 3–13 ml eluates, 20α-hydroxyprogesterone is in the 13–32 ml eluates and 17α-hydroxyprogesterone from 20–50 ml of the eluates.

Standard Curve

A range of 0, 0.5, 1, 2, 5, 10 and 20 ng progesterone in 50 µl of ethanol in 3 ml test-tubes was evaporated to dryness. ³H-corticosterone-CBG mixture (1 ml) containing 27–32 ng ³H-corticosterone and 100 µl CBG plasma in 100 ml water was added to each tube and incubated at 45°C for 5 min to facilitate the progesterone into solution. After shaking on a vortex mixer, the tubes were incubated in an ice bath for 30 min to facilitate binding by CBG. With the tubes still in the ice bath, 40 mg activated Florisil was added to the first tube which was then shaken on a vortex mixer for exactly 30 seconds. The tube was returned to the ice bath. The process was repeated until all the tubes had been treated with Florisil. Then 0.5 ml

of the supernatant was transferred into a polyethylene counting vial and 5 ml of scintillation fluid added. The samples were counted with a Packard Tri-Carb model 3320-01 liquid scintillation spectrometer for 10 min.

Subjects

Blood was collected in the morning from 206 normal pregnant patients at various stages of gestation. Five serum specimens at different trimesters were extracted and chromatographed as described above and 1 ml eluants were assayed against a progesterone standard. Thereafter, all 206 sera were extracted with the same batch of petroleum ether and assayed directly without subsection to prior column separation.

Results

Sensitivity, Specificity and Accuracy

The typical standard curve under the conditions of the assay procedures is presented in Figure 1. It has a working range of 0.5 to 10 ng/ml. Although 0.1 ng/ml could be dis-

tinguished from 0 ng/ml, for the purpose of assaying pregnancy samples the 0.1 to 0.5 ng/ml range was not required.

CBG binds a wide variety of steroids and displacement curves produced by testosterone, 20 α -hydroxyprogesterone, 17 α -hydroxyprogesterone and corticosterone are shown in Figure 1. This allows the competitive protein binding method to be modified to measure other steroids simply by a selective choice of solvents for extraction.

The standard elution patterns of the petroleum ether extracts of 0.5 ml of first and third trimester pregnancy sera are shown in Figure 2. More than 85% of the extracted steroid is progesterone, whilst 20 α -hydroxyprogesterone and 17 α -hydroxyprogesterone together accounted for less than 15%.

Recovery of ^3H -7-progesterone added to 12 samples of male serum ranging from 1 to 100 ng/ml was found to be $75.9 \pm 3.5\%$. To

Fig. 1. Standard Curve for Progesterone Assay. Only values within the 0.5–10 ng range are read. Note the displacement curves of ^3H -corticosterone-CBG system produced by testosterone, 20 α -hydroxyprogesterone, 17 α -hydroxyprogesterone and corticosterone.

Fig. 2. Steroid fractions from petroleum ether extracts of 0.5 ml of a first trimester (10th week) pregnancy serum (A) and a third trimester serum (B). The major peaks are progesterone and the minor peaks are 20 α -hydroxyprogesterone. There is a small amount of 17 α -hydroxyprogesterone in the first trimester.

exclude possible error which may result from quenching, ether extracts of 0.1 ml male serum was added to the standard tubes and

dried off before the addition of the scintillation fluid. All figures were corrected for extraction loss. Throughout the study, approximately 90% of the tested samples gave duplicates which were within $\pm 10\%$ of their mean values. The remainder were reassayed.

Effect of Incubation Time

Standard curves performed with 10, 20, 40 and 60 min incubation in the ice bath showed no significant alteration to the sensitivity of the assay. There was also no significant increase in binding of ^3H -corticosterone to CBG when the incubation time was prolonged beyond 20 min.

Effect of Incubation Temperature on the Standard Curve

To maintain the incubation temperature evenly at 4°C throughout the assay the tubes were immersed in the ice shavings to a depth of 5 cm. When the temperature was not properly maintained at 4°C, the standard curve was severely flattened (Fig. 3).

Florisil Contact Time

The percentage of ^3H -corticosterone-CBG removed by activated Florisil increased directly when the mixing time was increased (Fig. 4). Reproducibility was achieved by observing an exact 30 second contact time with Florisil for every tube in the assays.

Serum Progesterone in Normal Pregnancy, Hydatidiform Mole and Choriocarcinoma

The serum progesterone concentrations of random single samples collected from 250 pregnant Asian patients is shown in Table 1. In the first trimester the mean value of 19.1 ng/ml was only slightly higher than the level during the luteal phase of the menstrual cycle. It increased markedly to 60.4 ng/ml in the mid trimester and 135.3 ng/ml in the last trimester. At term, mean serum progesterone was 156.9 ng/ml. There is a wide difference between the lowest and highest values for serum progesterone all throughout pregnancy.

Fig. 3. Effect of incubation temperature on the standard curve. Continuous line shows standard curve with the tubes properly immersed in ice shavings. Broken line represents a standard curve in an experiment where the tubes were seated in a styrofoam box with ice cubes and the temperature was not properly maintained.

In 21 cases of hydatidiform mole, mean serum progesterone was 106.0 ng/m/ which is in the range of levels normally encountered in the last trimester. However, the duration of amenorrhoea was generally between the 10th to 22nd week. Hence, mole patients showed elevated serum progesterone. In 10 cases of choriocarcinoma, the mean serum progesterone was 16.9 ng/m/. The range of values corresponded to those in the first trimester.

Fig. 4. Percentage of ^3H -corticosterone-CBG removed by Florisil with various mixing times.

Table 1. Serum progesterone in normal pregnancy, hydatidiform mole and choriocarcinoma

	No. of Samples	Mean (ng/m/)	Range (ng/m/)
Normal Pregnancy			
1st Trimester	87	19.1	3.3 — 51.3
2nd Trimester	39	60.4	17.4 — 115
3rd Trimester	80	135.3	39.5 — 334
Hydatidiform Mole	21	106.0	17.8 — 263
Choriocarcinoma	10	16.9	1.3 — 61

Discussion

The competitive protein binding method is very simple to carry out and can be rapidly repeated in any laboratory. The important factors to note in the method are a strict temperature control and an exact contact period with Florisil. The petroleum ether lot should be carefully selected and the Florisil must be washed several times, activated and maintained in a dry state.

The values of serum progesterone in this study are similar to the plasma values reported by Johansson using a similar method (Johansson, 1969), and are comparable to the values obtained by gas liquid chromatography (Yannone, 1968) and the double isotope

derivative assay (Weist, 1967). The results also correspond to the findings of earlier workers who employed a chemical method (Short, 1958; Greig *et al.*, 1962).

The CPB method has the advantage of simplicity, sensitivity, speed and economy. In our laboratory, a single technician handles 50 to 100 samples per week. The original bioassay methods had a low accuracy, required large numbers of laboratory animals and the results were only available after 44 hr. The chemical method required a large blood sample of 40 ml (Short, 1958) and both gas liquid chromatography and double isotope derivative assay are difficult to perform. An advantage of the present technique is the exclusion of the chromatographic step which was made possible by the choice of suitable petroleum ether solvent. By running chromatograms on the petroleum ether extracts, it is possible to select petroleum ether lots which will selectively extract progesterone without carrying over appreciable amounts of 20α -hydroxyprogesterone and 17α -hydroxyprogesterone. However, during pregnancy, progesterone is the principle progestogen present in the circulation. 20α -hydroxyprogesterone present in low concentration in the plasma has a low affinity for CBG and runs parallel to progesterone throughout pregnancy and therefore does not appreciably affect the serum progesterone value (Greig *et al.*, 1962; Rubin *et al.*, 1970).

In hydatidiform mole, ovarian theca lutein cysts secrete appreciable quantities of 20α -hydroxyprogesterone and 17α -hydroxyprogesterone (Teoh *et al.*). Elution of petroleum ether extracts of sera from such patients showed that the two hydroxyprogesterones taken to-

gether accounted for approximately 15% of the serum "progesterone" measured on an uneluted petroleum ether extract. For clinical purposes, this may probably be ignored when a rapid result is required.

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