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A COMPETITIVE PROTEIN-BINDING METHOD FOR THE MEASUREMENT OF PROGESTERONE IN HUMAN AMNIOTIC FLUID

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SUMMARY

A simple method is described for the estimation of progesterone in human amniotic fluid. The method is specific, accurate and rapid enough to allow the measurement of 24 samples in duplicate in one day. Replicate analyses of a single sample of amniotic fluid gave a coefficient of variation of 4·11%. The method could also be applied to other biological fluids such as plasma.

INTRODUCTION

METHODS are available for the rapid analysis of the contents of human amniotic fluid. Various parameters including optical density have been investigated to determine their relationship to the functional status of the feto-placental unit[1]. Suggestive evidence has been obtained to show that amniotic fluid estriol could serve as a good index of feto-placental function[2, 3]. Other steroids have not been investigated to the same extent.

Progesterone is an important steroid for the maintenance of pregnancy. If amniotic fluid contents faithfully mirror the metabolic activity of the fetus[4], then alterations in activity may be reflected in the steroid profile. Thus, the analysis of progesterone in amniotic fluid might prove to be a valuable aid in assessment of feto-placental function. Two methods have previously been used to determine the concentration of progesterone in pooled amniotic fluid—a double isotope derivative method[5] and gas–liquid chromatography[2, 4, 6]. Both of these methods are somewhat tedious and time consuming. More rapid methods such as competitive protein binding radioassay and radioimmunoassay are now available. It was the purpose of this investigation therefore, to develop and apply a method for the estimation of progesterone in human amniotic fluid.

MATERIALS AND METHODS

Carrier steroids: progesterone, testosterone, 20α-dihydroprogesterone, 17α-hydroxyprogesterone, estradiol and estriol were obtained from Steraloids Inc., Pauling, New York. They were recrystallized and melting points determined prior to use. Progesterone-[1,2-^{3}H] (50·3 Ci/mmol), corticosterone-[1,2-^{3}H] (50 Ci/mmol), 17α-hydroxyprogesterone-[1,2-^{3}H] (49·2 Ci/mmol), 20α-dihydroprogesterone-[1,2-^{3}H] (33·5 Ci/mmol) and testosterone-[1,2-^{3}H] (45 Ci/mmol) were obtained from New England Nuclear, 575 Albany Street, Boston, Mass. 02118. Purity of these radioactive standards was checked by paper chromatography of an

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aliquot in either n-heptane: methanol: water: (5:4:1 by vol). (system A) or n-heptane: benzene: methanol: water::66 (34:80:20 by vol). The scintillation materials 2,5-diphenyloxazole (PPO), *p*-bis [2-(5-phenyloxazolyl)]-benzene (POPOP), and dimethyl POPOP were obtained from Packard Instruments Co., Downer's Grove, Illinois. A Packard Tri-Carb Model 3375 liquid scintillation spectrophotometer was used for the measurement of radioactivity. Scintillation fluid was prepared with one part Triton X-100 (Packard Instruments Co.) and two parts toluene scintillation solution composed of 8·25 g PPO and 0·25 g dimethyl POPOP per l. Ten ml of this mixture was used for each aqueous sample. In some instances scintillation fluid was made with PPO and POPOP in toluene. Radioactive areas on chromatograms were located using a Packard Model 7200 radiochromatogram scanner.

Toluene, n-hexane, methanol, ethanol, n-heptane, benzene, florisil (60–100 mesh), boric acid and sodium hydroxide from Fisher Scientific Co., Don Mills, Ontario were of reagent grade quality. Solvents were redistilled prior to use. Water was de-ionised and redistilled in an all glass apparatus.

Stock solutions of 1 mg steroid per ml in ethanol were generally prepared. Diluted solutions were not used for more than 1 month. Prior to use the corticosterone-[1,2-^{3}H] was diluted with carrier progesterone to give an activity of approximately 60 μCi/μg. Borate buffer was prepared by making a 0·05 M solution of boric acid and adjusting to pH 7·6 with 10 N sodium hydroxide. Dog plasma was obtained from a healthy "Labrador" and stored in 1 ml aliquots at −15°C. The binding ability of the plasma was checked and only that which bound more than 50% of labelled corticosterone was used.

Florisil was washed with distilled water and methanol and then dried overnight at 100°C before use. Whatman No. 40 ashless paper washed with methanol and methanol: benzene: (1:1) (Fisher Scientific Co.) was used for chromatography of some extracts in system A. In these cases about 1000 cpm [^{3}H]-progesterone was added as a recovery marker prior to extraction of 1 ml amniotic fluid. Standards were located by their absorption of ultraviolet light. Corresponding areas were eluted with 2 ml methanol using the syringe technique [7]. The eluates were dried under nitrogen, a 40% aliquot taken for recovery and 20% for assay.

PREPARATION OF BINDING GLOBULIN

Sephadex G-25 fine (Pharmacia Ltd., Montreal, Quebec) was soaked overnight in borate buffer. This was then packed in a glass column 1 cm dia., plugged with glass wool, to a height of 6 cm. Air bubbles were removed by agitation and the column washed with borate buffer at room temperature. One ml of dog plasma was then added to the top of the column and washed through with borate buffer. As the dark band of protein approached the bottom of the column it was collected in a 50 ml volumetric flask in which had been dried 2 μCi of the diluted corticosterone-[1,2-^{3}H]. Immediately after the dark band is eluted the volumetric flask is removed and made up to 50 ml with borate buffer. This was mixed, incubated at 47°C for 15 min and then stored overnight at 4°C before use (CBG solution). This solution contained 40 nCi/ml corticosterone. The Sephadex column could be washed with borate buffer and stored at 4°C for repeated use.

SAMPLE EXTRACTION

Amniotic fluid (0·1 ml) was pipetted in duplicate with an Eppendorf micro-

pipette (Brinkman Instruments, Rexdale, Ontario) into 5 ml glass stoppered "Quickfit" tubes and diluted to 1 ml with distilled water. Distilled water alone was also used as blanks. Three ml of n-hexane was added and the tubes shaken manually for 1 min. The organic layer was transferred with Pasteur pipette into 12 × 75 mm disposable glass tubes and dried under nitrogen at 47°C. One more extraction was carried out and dried. Steroid standards in the range 1–20 ng were also dried at the same time. The dried extracts and standards were then assayed by the protein binding method.

ASSAY

Five hundred μl of the radioactive CBG solution were added by means of an Eppendorf pipette, to the residue in each tube. The samples were mixed on a Vortex mixer and incubated in a water bath at 47°C for 10 min. They were mixed again on the Vortex mixer at the end of the incubation period and placed in the cold room, 4°C, for at least 40 min. In the cold, florisil, 40 mg, was added to each sample individually with a plastic spoon to absorb the unbound labelled steroid. The rack containing all the samples was then shaken vigorously by hand for exactly 1 min, and the florisil allowed to settle for 5 min. Aliquots of 0·25 ml containing the bound steroid were then pipetted from each tube into scintillation vials and counted in the Triton X-100 solution. The efficiency of counting was about 37%.

RESULTS AND DISCUSSION

Figure 1 consists of 33 separate standard curves with the mean and standard deviation for specific amounts of progesterone. The coefficients of variation at 1, 2, 5, 10 and 20 ng were 4·72, 4·30, 4·82, 6·92 and 12·60 respectively. The water blank on 40 assays gave a mean of 0·27 ± 0·15 S.D. ng/ml. The percent radioactivity bound in the tube without standard was usually 50–70%. Six separate assays on a single sample of amniotic fluid gave 43·8 ± 1·8 ng/ml. The coefficient

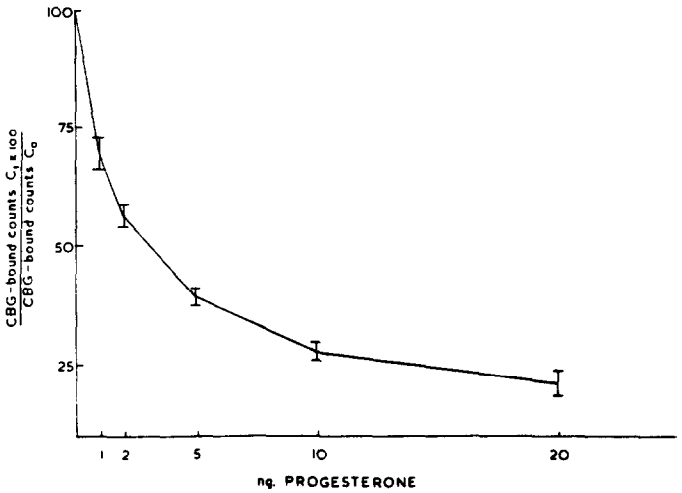

Fig. 1. Standard curve of bound activity against increasing concentrations of pure standards. This is a composite graph of 33 runs with the mean and standard deviation plotted for each value. CBG-bound radioactivity in standards (C_1) is expressed as a percentage of that bound by CBG in tubes without standard (C_0).

of variation was 4·11%. In Table 1 are shown the recoveries of ^{3}H-progesterone added to samples of amniotic fluid. Since these were quite high an internal standard was not added to samples to correct for losses.

A large number of steroids such as cortisol, corticosterone, 17α-hydroxyprogesterone and testosterone can be measured with the protein solution. However, n-hexane would only extract unconjugated nonpolar steroids and most of the polar competing steroids would remain in solution. In addition, steroids possessing a hydroxyl group such as cortisol are probably all conjugated. Further assessment of specificity was carried out by extracting 6 separate samples of amniotic fluid with diethyl ether. The extracts were then chromatographed on paper with system A which separated progesterone from all other competitive steroids such as corticosterone and 17α-hydroxyprogesterone. Other steroids which have an R_F lose to progesterone in this system will not bind to CBG. When the results were analyzed for variance, the regression line was represented by $Y = 0{\cdot}91x + 1{\cdot}84$. Since there was a significant correlation between the two series of values assays were carried out directly on the n-hexane extracts. Competing steroids account for less than 10% of the measured progesterone using this method.

Table 1. Recovery of [^{3}H]-progesterone added to diluted amniotic fluid

Sample No.	% Recovery
1	97·7
2	100·1
3	95·6
4	97·2
5	99·4
6	98·6
7	100·8
8	100·1
9	97·6
10	97·7

Mean = 98·48.
S.D. = 1·53.

The accuracy and precision of the method were determined by recoveries of known quantities of authentic progesterone from a pool of amniotic fluid which contained 20 ng progesterone/ml. Table 2 shows the recovery after correction for progesterone concentration in the original pool. The inter-assay coefficient of variation on the same sample of amniotic fluid was 4·11%. Since it was not possible to obtain a progesterone-free pool of amniotic fluid recoveries of small amounts of progesterone would be variable.

Analysis of serial samples using the direct assay on the n-hexane extracts of amniotic fluid from three patients showed a progessive fall as depicted in Fig. 2. These levels of progesterone are similar to those found by other workers [4–6] who used gas–liquid chromatography and isotope derivative methods on pooled amniotic fluid. It is of interest that the concentrations tend to fall towards the end of pregnancy. The protein binding method of Lurie and Patterson [8] was tried

Table 2. Accuracy of method. Recovery of progesterone added to diluted amniotic fluid

Progesterone added (ng)	No. of samples	Progesterone recovered [ng (mean)]	S.D.
5	20	6·10	0·68
10	20	11·80	3·05
20	24	23·20	2·50

$Y = 1{\cdot}2x + 0{\cdot}2$.
$r = 0{\cdot}9998$.

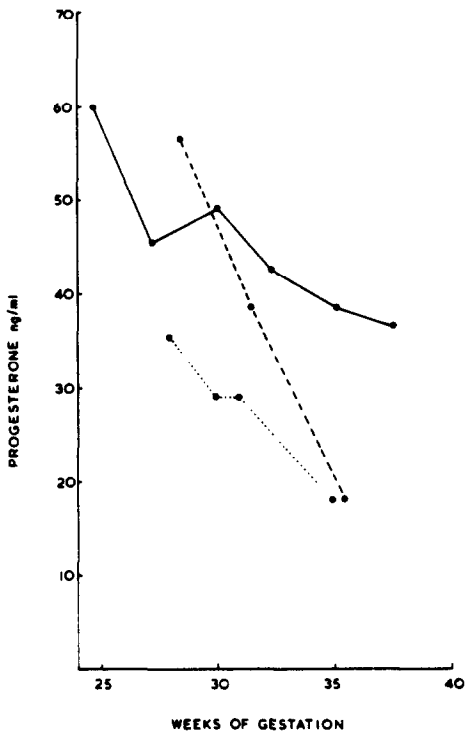

Fig. 2. Concentration of progesterone in amniotic fluid taken serially from three pregnant subjects.

but found to be unsatisfactory in our hands for amniotic fluid. In view of the difficulties encountered by investigators in setting up the competitive protein binding method [9] it is possible that different conditions must be tried in the laboratory to ascertain which are optimal. Differences and difficulties may therefore be due to unexplained environmental conditions.

The present method can also be used to measure progesterone in non-pregnancy plasma. Data from such a study are shown in Fig. 3. The levels of progesterone during the follicular phase of the cycle are negligible. They increase only after ovulation and are in agreement with other published data. The results of the determination on amniotic fluid show that the method can be used to measure progesterone in this fluid. Progesterone levels in amniotic fluid have

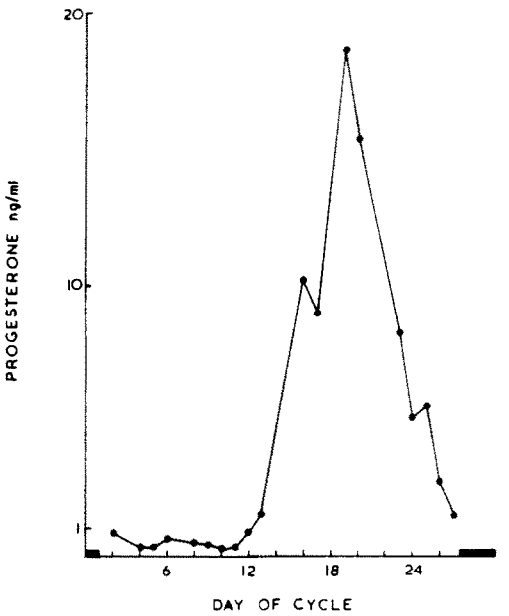

Fig. 3. Levels of progesterone in peripheral plasma during a normal menstrual cycle.

recently been reported[10, 11]. Whether the progesterone in amniotic fluid has any physiological significance is a matter of speculation and more detailed studies would be needed to clarify this question.

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