The Polarographic Estimation of Steroid Hormones

5. DETERMINATION OF PROGESTERONE IN BLOOD

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A reliable method for the determination of progesterone in blood would appear to have great value in both chemical and physiological fields. The common biological methods for the assay of this hormone are, with one exception, of relatively low sensitivity, requiring amounts of the order of 1 mg. for a definite response. In consequence, most of our knowledge of the metabolism of progesterone has been derived from studies of the excretion of the metabolite, pregnane-3α:20α-diol. Such studies suffer from the disadvantage that the urinary pregnanediol level will be influenced by liver and kidney function as well as by endogenous progesterone production. The value of a direct method which can be applied to blood is thus evident.

Haskins (1941) adapted the intrauterine injection technique of McGinty, Anderson & McCullough (1939) to the problem of the determination of progesterone in pregnancy blood. He used progestational changes in the uterus as end point, following the classical method of Corner & Allen (1929). He examined twenty pregnancy bloods and only in one case was able to detect progesterone, equivalent to 0·13 μg./ml. serum. This was at the bottom limit of sensitivity of the bioassay used. Hooker & Forbes (1947) developed a modified McGinty bioassay using the mouse as test animal and histological changes in the endometrial stromal nuclei as end point. The method was claimed to detect progesterone at a concentration of 0·3 μg./ml. Hooker & Forbes (1949*b*) later examined the specificity of the method. They found that, of the twenty-four steroids tested, progesterone alone gave the characteristic response. Application of the method to human pregnancy blood (Hooker & Forbes, 1949*a*) indicated a value of 5·3 μg./ml.; 90 % of this was in the free state, the remaining 10 % requiring hydrolysis to render it biologically active. Hooker & Forbes (1950) also applied the method to follow progesterone concentrations during the menstrual cycle of the monkey.

A chemical approach to the problem was made by Reynolds & Ginsburg (1942), who developed a method based on the ultraviolet absorption of extracts at 240 mμ. Haskins (1950) applied a similar method to pregnancy blood. In agreement with his earlier bioassay experiments and in contradiction to Hooker & Forbes (1949*a*), he was unable to detect any progesterone in the blood examined, although his method could detect 0·1 μg./ml. plasma. He also demonstrated that progesterone injected intravenously in the rabbit disappeared from the circulation very rapidly and that the disappearance rate was decreased by hepatectomy.

Our own interest in the problem was stimulated by the work of Hooker & Forbes (1949*a*). The levels found by them should be capable of polarographic determination as Girard complexes of Δ^4-3:20-diketones by the technique of Barnett, Henly & Morris (1946). A method has been developed based on extraction and partition between organic solvents, with final separation of the individual steroids by partition chromatography and subsequent polarographic estimation. A preliminary account of the work has appeared previously (Butt, Morris & Morris, 1949).

EXPERIMENTAL

Determination of progesterone in blood. Blood used in the procedure was collected in tubes containing heparin and worked up without delay.

Extraction and preliminary fractionation. The solvent partition method used is shown schematically in Fig. 1 and illustrated by the following example. Plasma (20 ml.) is poured slowly into 100 ml. of a 3:1 (v/v) ethanol-ethyl ether mixture with mechanical stirring. The mixture is stirred for a further 10 min., centrifuged and the supernatant liquid separated. The precipitate is washed twice by stirring with 50 ml. portions of the 3:1 ethanol-ethyl ether mixture. The combined extracts are concentrated *in vacuo* to a volume of 20 ml. and diluted with 40 ml. of water. The aqueous ethanol mixture is extracted three times with 60 ml. portions of ethyl acetate (A.R., redistilled). The ethyl acetate extracts are evaporated to dryness *in vacuo* at a bath temperature below 50°. The residue is dissolved in 70 % aqueous methanol (A.R., redistilled) by warming, and transferred quantitatively to a 25 ml. flask, a total volume of 10 ml. being used. The mixture is stored for 18 hr. at −15°. The aqueous methanol is separated from the precipitated lipids by centrifugation in an angle centrifuge at 5000–6000 rev./min., the process being carried out in a refrigerator at −15°. The supernatant liquid is diluted with 20 ml. of water and extracted three times with 30 ml. portions of light petroleum (b.p. 40–60°; A.R., redistilled). The combined light petroleum extracts are washed twice with 30 ml. portions of water and evaporated to dryness *in vacuo* below 50°. The residue is

transferred quantitatively to a small tube with 1·0 ml. absolute ethanol, the ethanol removed *in vacuo* and the residue dissolved in 0·1 ml. 70% (v/v) methanol for transfer to the partition column. All solvents used in this procedure should be redistilled and a control experiment carried out to ensure that the polarographic blank is satisfactory, i.e. is indistinguishable from that of the base solution.

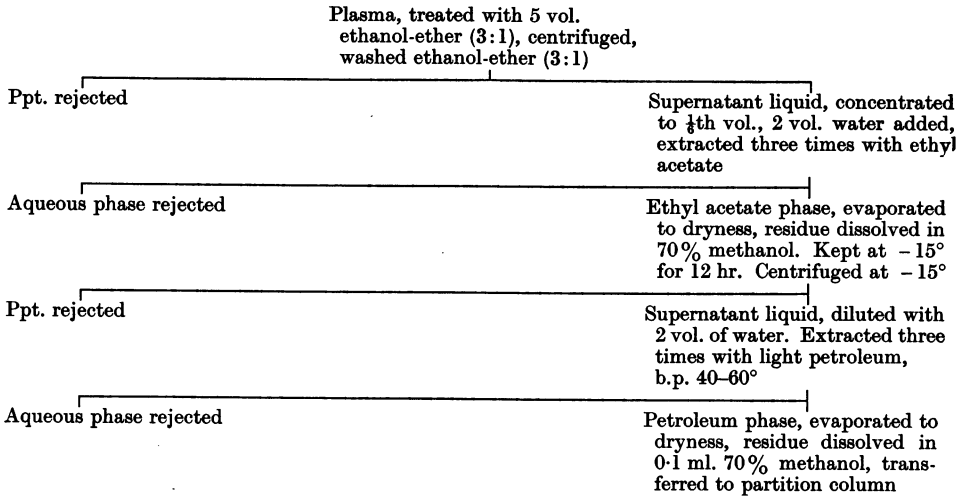

Fig. 1. Preliminary partition system for separation of progesterone from blood.

Partition chromatography. Columns used have been 6 mm. in diameter and 6 cm. long. The glass tube should be about 15 cm. long, provided with a capillary outlet and a B14 standard joint at the upper end. For preparation of the column a Martin packer (Howard & Martin, 1950) is used. This consists of a stainless steel disk of diameter about 1 mm. less than that of the column. The disk has six to eight holes, 0·7 mm. in diameter and a long, central, stainless steel handle. Hyflo grade Supercel has been used as supporting phase throughout this work. Hyflo Supercel (50 g.) is treated with conc. HCl (200 ml.) and allowed to stand overnight. The mixture is heated at 100° for 1 hr., cooled and washed with water until the washings are neutral. The Supercel is finally air-dried at 110°. Aqueous methanol (70%) was used as stationary phase and *n*-hexane as mobile phase. The two phases should be very thoroughly equilibrated before use. Supercel (3 g.) is treated with 2 ml. stationary phase added in small portions with thorough mixing. The Supercel will still appear almost dry. Sufficient mobile phase is added to make a slurry. A small piece of absorbent cotton wool is forced down the column to the capillary outlet. A small portion of the Supercel slurry is transferred to the column with excess mobile phase and forced down with the Martin packer. This is moved rapidly up and down to give a uniform suspension. The packer is then moved down slowly to trap some of the Supercel under the disk and to pack it down firmly. The rest of the Supercel in suspension is then packed down in a similar manner. The operation is repeated by addition of further Supercel slurry to the column and continued until the required length is reached. Care should be taken that there is always some mobile phase above the Supercel and that no air bubbles are trapped during packing. A well packed column should appear quite uniform and not show any marked transverse layering. The column is finally washed with about 10 ml. of mobile phase. A column 6 × 0·6 cm. should flow under gravity alone at about 1 ml./hr. Excess mobile phase is removed from the top of the column with a capillary pipette and the liquid level forced down under 1–2 cm. Hg positive pressure until it is about 1 mm. below the surface of the Supercel. Sufficient dry Supercel is now added to form a layer 1–2 mm. deep on the top of the column. The sample to be examined, dissolved in about 0·1 ml. stationary phase, is added to the dry Supercel, with a capillary pipette, and forced down under slight positive pressure. Mobile phase is now added carefully with a capillary pipette so that the top of the column is not disturbed. A separating funnel with standard joint, containing mobile phase, is next attached. This is provided with a capillary inlet so that it serves as a constant pressure vessel. The column is run and fractions collected as required.

The efficiency of packing may be tested on a 70% methanol-*n*-hexane column using Sudan III in *n*-hexane as test substance. The dead space in a 0·6 × 6 cm. column should be about 0·6 ml. (see p. 436).

Polarographic estimation. With the column dimensions given the first 1 ml. of eluate will contain no progesterone and is rejected. The second and third ml. of eluate, which contain the progesterone if present, are combined and evaporated to dryness *in vacuo* at a temperature not exceeding 50°. The residue is finally dried *in vacuo* over P_2O_5 overnight. The residue is treated with 0·02 ml. of a freshly prepared solution of Girard's reagent T (20 mg.) in anhydrous acetic acid (0·2 ml.) and heated for 2 min. at 100°. The reaction product is treated with 2 ml. of the polarographic base solution (0·5 N-NaCl, 1 vol.; 0·2 N-NaOH, 2 vol.; water, 1 vol.), transferred to the polarographic cell, and O_2 removed with a stream of N_2. General polarographic technique and purification of reagents follow the description of Barnett *et al.* (1946).

A standard curve is constructed using 5–20 μg. amounts of progesterone at sensitivity 4 μa. = 100 scale divisions and 2 μa. = 100 scale divisions of the Tinsley polarograph.

RESULTS

Separation of individual steroids on partition columns

The separation of progesterone, androst-4-ene-3:17-dione, deoxycorticosterone and testosterone with different compositions of stationary phase is shown in Fig. 2. As a result of these experiments 70% aqueous methanol was chosen as stationary phase as it permits of a clear separation of progesterone from androst-4-ene-3:17-dione, which appears to behave most like progesterone among the naturally occurring Δ^4-3-ketosteroids. The symmetry of the elution curves suggests that the separation mechanism is mainly partition. This was tested by comparison of the partition coefficients calculated from the elution curves with those measured directly.

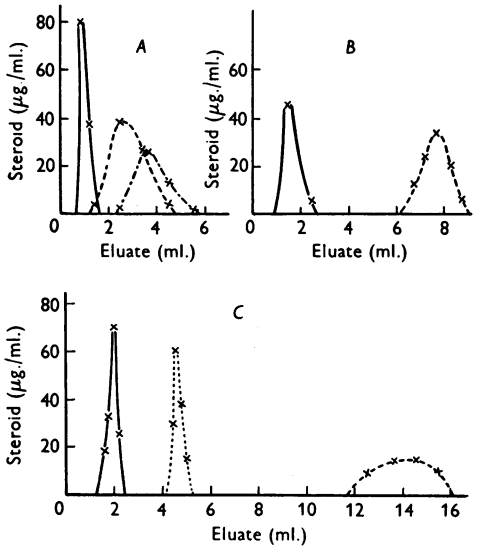

Fig. 2. Separation of steroids on partition columns. *A*, 40% methanol-*n*-hexane column; *B*, 60% methanol-*n*-hexane column; *C*, 70% methanol-*n*-hexane column. Progesterone, ——; androst-4-ene-3:17-dione, ...; deoxycorticosterone, – – –; testosterone, –·–·–.

The partition coefficients were calculated from the elution curve maxima by a modification of the equation of Martin & Synge (1941). If A = cross-sectional area of the column (sq.cm.), A_S = cross-sectional area of the stationary phase (sq.cm.), A_L = cross-sectional area of the mobile phase (sq.cm.), L = length of column (cm.), K = partition coefficient = (concentration of solute in stationary phase)/(concentration of solute in mobile phase), R = (rate of band movement on column)/(rate of liquid surface movement above column), then

$$K = \frac{A}{RA_S} - \frac{AL}{A_S}. \quad (1)$$

If V_r = the retention volume = volume of eluate to peak concentration (ml.), the band maximum will have moved through the length L of the column when the retention volume V_r has passed through. Thus

$$R = AL/V_r. \quad (2)$$

Substituting for R in equation (1)

$$K = \frac{V_r}{LA_S} - \frac{A_L}{A_S}. \quad (3)$$

The values of V_r for the steroids studied are given in Table 1.

Table 1. *Retention volumes of steroid ketones on Supercel columns with* n-*hexane as the mobile phase and aqueous methanol as the static phase*

Steroid	Concn. of methanol (%)	Retention volume (ml.)
Progesterone	40	0·94
	60	1·6
	70	2·0
	80	3·5
Androst-4-ene-3:17-dione	70	4·8
Deoxycorticosterone	40	2·5
	60	7·7
	70	14·4
Testosterone	40	3·6

A_S was determined from the dimensions of the column and the volume of stationary phase used. A_L was determined from the column 'dead volume', which is equal to the retention volume of a solute of zero partition coefficient. The dye Sudan III was used for this purpose; 'dead volume' determinations were carried out on the columns immediately after the steroid experiments. Values were from 0·65 to 0·70 ml.

Table 2. *Comparison between partition coefficients measured directly and calculated from column data*

(See Eqn. 3 and Table 1. Mobile phase *n*-hexane.)

Substance	Stationary phase (% methanol)	K (concn. stationary phase/concn. mobile phase) Calc.	Direct
Progesterone	40	0·39	0·42
	70	1·95	1·95
Deoxycorticosterone	40	2·76	2·66
	70	19·6	23·5
Androst-4-ene-3:17-dione	70	6·0	5·6

Partition coefficients were determined directly by equilibrating the steroid between the two phases and determining the concentrations in both phases polarographically. Comparisons of the two values for K for some steroids with different phase concentrations are given in Table 2. The agreement over a wide range of K values indicates that the column mechanism is essentially partition.

The number of theoretical plates in the columns used were calculated for some of the elution curves using the equation of Matheson (1949). Values of sixty to eighty theoretical plates for a 6 cm. column were obtained. The height equivalent to a theoretical plate was thus rather less than 1 mm.

Recovery of progesterone from plasma

Some typical recoveries of progesterone added to 20 ml. samples of plasma, which was then taken through the routine process, are given in Table 3. It will be seen that even at the 10 μg. level recoveries are satisfactory. 1 μg. can easily be detected.

Table 3. *Recovery of progesterone added to plasma*

(20 ml. plasma used.)

Progesterone added (μg.)	Recovery (%)
10	85
10	91
10	89
20	94
20	89
20	86
30	90
30	96
30	92

Recovery of progesterone from circulating blood

Normal rats. In order to test the method more rigorously and to obtain an estimate of the rate of disappearance of progesterone from the circulation, progesterone (1–3 mg. in 0·4 ml. 50% aqueous ethanol) was injected intravenously into normal rats and blood samples for analysis taken 5 min. later. Results are given in Table 4. It will be seen that more than 95% of the injected progesterone has been removed from the circulation in 5 min.

Rats with ligated livers. Since Haskins (1950) has shown that hepatectomized rabbits eliminate progesterone from the circulation rather more slowly than normal animals, it was considered of interest to carry out similar experiments in the rat. The hepatic vein and artery were clamped and the animals left 10 days for shunting anastomoses to develop. The hepatic vein and artery were finally ligated before injection. This procedure minimizes trauma and formation of blood depots. The results are shown in Table 4. The recoveries obtained are only slightly higher than those obtained with normal animals.

Plasma progesterone in pregnancy

Blood samples of 12–20 ml. from thirteen women with pregnancies from 12 weeks to full term, were examined by the routine method. In no case was any progesterone detected. In one case the plasma extract was subjected to acid hydrolysis by the method of Hooker & Forbes (1949*a*) prior to the routine fractionation process. In this case also, no progesterone was detected. Blood from a woman suffering from infective hepatitis and with a pregnancy of 16 weeks' duration was also examined. A blood level of 0·4 μg. progesterone/ml. of blood was found here.

Progesterone in human placental blood

The routine method was also applied to human placental blood obtained from the umbilical cord. The results are given in Table 5. In two cases progesterone was detected, and in two further cases (nos. 3 and 4) a substance giving a polarographic wave resembling that characteristic of Δ^4-3-ketosteroids but showing no 20-ketosteroid wave was detected. The significance of this cannot at present be evaluated.

Table 4. *Rate of elimination of progesterone from the circulation of rats with normal and impaired liver function*

(Recoveries calculated on a blood volume of 6 ml./100 g. body weight.)

Rat no.	Body wt. (g.)	Progesterone injected (mg.)	Sampling time (min.)	Recovery (%)
		Normal rats		
1	190	1	5	1·4
2	195	2	5	4·7
3	200	3	5	2·4
4	200	1	9	0·0
		Rats with ligated livers		
1	240	3	5	3·7
2	270	3	5	3·2
3	280	3	5	3·9

Table 5. *Estimation of progesterone in placental blood*

No.	Sex of infant	Progesterone (μg./ml.)	Remarks
1	F	1·3	Normal } biovular
2	F	0·3	Small } twins
3	M	0	Normal
4	M	0	Normal
5	F	0	Arterial blood only

DISCUSSION

The results of the present work show that the amounts of progesterone present in blood obtained from cases of pregnancy of varying duration were in all cases below 0·1 μg./ml., the limit of sensitivity of the method with sample volumes of 20 ml. of plasma. This is in agreement with the findings of Haskins, using both biological and physico-chemical methods. Dr K. Dobriner (Sloan-Kettering Institute, New York) has also informed the authors privately that he was unable to isolate any progesterone from several litres of pregnancy blood using the methods of fractionation and infrared spectroscopy developed in his laboratory. These findings are in conflict with those of Hooker & Forbes (1949*a*) obtained by their intrauterine injection bioassay technique. It thus appears likely that the substance responsible for the high plasma 'progesterone' values of the Hooker & Forbes method is not in fact progesterone, although the possibility of an augmentation effect in the bioassay produced by some unknown factor in blood in conjunction with plasma progesterone levels below 0·1 μg./ml. cannot be excluded.

The adequacy of the present technique under physiological conditions appears to be substantiated by the detection of progesterone in the hepatitis case and in placental blood as well as by the rat experiments.

The value of the partition column method for the quantitative separation of steroids is well illustrated by this work. The chief advantages appear to be: (1) symmetrical elution curves at the concentration used, obviating losses through 'tailing'; (2) independence of the properties of the solid phase, in marked contrast to adsorption chromatography; (3) simple calculation of retention volumes from partition data, and of column dimensions necessary for a given separation; (4) the possibility of continuous variation of column behaviour by alteration of the stationary phase.

SUMMARY

1. A method for the determination of progesterone in blood, based on solvent partition, partition chromatography and polarographic determination as a 3:20-ketosteroid, is described.

2. The concentration of progesterone in the circulation after injection into normal and partially hepatectomized rats has been examined.

3. Application of the method to human pregnancy blood has shown that the progesterone level is less than 0·1 μg./ml.

4. Progesterone has been detected in human placental blood.

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