# A MODIFIED PROCEDURE FOR THE MEASUREMENT OF PLASMA PROGESTERONE BY RELIANCE ON PROTEIN BINDING

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## SUMMARY

A modified procedure for the assay of progesterone in biological fluids is presented.

After addition of a tracer dose of radioactive progesterone, extraction of the sample and chromatography on thin layer plates, the extract is processed through a mini-column made out of Sephadex LH-20 to reduce interference in the final quantitation by protein-binding.

Many technical aspects thought to be of importance for reliable measurement and values obtained in well-documented situations are mentioned and discussed.

## INTRODUCTION

In VIEW of the difficulties met when attempting to adopt methods hitherto described for the assay of plasma progesterone by means of protein binding[1–4] we were forced to modify the procedure so as to render it more reliable. We thought it worthwhile to describe and discuss this modified, and hopefully improved, technique used for such measurements.

## REAGENTS AND MATERIALS

*Solvents.* All solvents were reagent grade (*pro analysi*, Merck, Germany) and were employed without further purification.

*Steroids.* [1.2-³H]progesterone (S.A. 33·8 Ci/mmol) and [1.2-³H]corticosterone (S.A. 49·5 Ci/mmol) were purchased from NEN (Frankfurt/Main, Germany) and used without further purification. The stock solution stored at 1°C (250 μCi in 100 ml of benzene containing 10% ethanol, v/v) of each radioactive steroid was tested monthly for radiochemical contaminants. Corticosterone keeps well for 6 months at least and progesterone for more than 15 months, if a contribution of impurities up to 5% is tolerated.

Unlabelled steroids were used without further purification after isotopic dilution and chromatographic control of purity.

*Materials.* Florisil (synthetic adsorbent 60/100 mesh Cat. No. 83790, obtained from Pleuger, Belgium) was treated as indicated by Neill *et al.*[1].

Thin-layer plates were obtained from Merck (Silica Gel F 254 precoated plates No. 5729/0050). They were employed as such after activation at 120°C for 20 min. Over a 1-yr period two or three different batches were used, all behaving similarly.

Glasswool, ('Fiberglass' Cat. No. 3950. from Owen–Corning Fiberglass Co.. Corning, N.Y., USA).

Sephadex LH-20 (Lot No. 2757) was used as obtained from Pharmacia. Uppsala, Sweden.

Evaporation of solvents was carried out in a heating block maintained at 70°C under a stream of air filtered through a Gelman membrane capsule filter No. 12101 (Gelman, Ann Arbor, Mich.. U.S.A.).

Disposable material: transfer pipettes, capillaries for t.l.c. and Sephadex LH-20 mini-columns were made out of Pasteur pipettes obtained from Harshaw (Utrecht, The Netherlands) and modified as indicated below. Disposable culture tubes: $85 \times 12$ mm (Vel, Belgium).

Florisil dispenser: as suggested by de Souza *et al.*[5], a 250 ml separatory funnel with a teflon stop-cork was used, one end of the hole and the necessary volume of the bore being sealed with 'Parafilm' (American Can Co., Wisc.. U.S.A.) to obtain the desirable capacity. The amount delivered by the dispenser was $33 \cdot 6$ mg $\pm 0 \cdot 2$ (S.D.).

Vortex Super-Mixer, Cat. No. 1291 (Lab Line Inst., Ill., U.S.A.).

*Handling of glassware.* Round-bottom stoppered tubes of different sizes were always washed in the standard washing-machine of the laboratory (Automatic G-19 Labor., Miele, Germany). The washing cycle includes: 2 successive washes and 4 rinses (Neo Disher 'N', Neo Disher 'LA', Hamburg, Germany). the last two being carried out with demineralized water. This kind of washing was adequate provided glassware was not allowed to dry after use: it was therefore kept immersed in a detergent bath (Alconox, N.Y.. U.S.A.) between use and automatic washing.

*Radioactivity counting.* Radioactivity was measured in a liquid scintillation spectrometer (Packard, Tricarb 3375) using the following mixture: PPO $8 \cdot 25$ g (2,5-diphenyloxazol), POPOP $0 \cdot 15$ g (2,2'-p-phenylen-bis-(5-phenyloxazol)) per liter of toluene. After dissolution, 500 ml of Triton X-100 (industrial grade) were added and a few minutes later 25 ml of distilled water were poured in while shaking the mixture. The resulting scintillation medium becomes transparent after mixing $0 \cdot 5$ ml of aqueous CBG with 6 to 12 ml of scintillator.

*CBG.* 'Corticosteroid Binding Globulin', was in fact plasma from women taking 100 $\mu$g mestranol daily for 2 months. The plasma was frozen in small aliquots, of which one at a time was thawed to make the 'CBG-solution' as follows: 4 $\mu$Ci [1,2-$^3$H]corticosterone as a dry residue taken up in 100 ml distilled water to which 140 $\mu$l of plasma were finally added. This solution was employed within 48 hr and kept at 4°C when not in use.

## PROCEDURE

*Extraction.* Approximately 1000 c.p.m. [1.2-$^3$H]progesterone in 100 $\mu$l ethanol were added to each of the extraction tubes and to a vial that served as a standard for a given series of samples. Then, plasma to be assayed was added and mixed, the amount depending on the expected concentration of progesterone: 4 ml of plasma were processed during the follicular phase of the menstrual cycle, after menopause, in men and before puberty in both sexes, and $0 \cdot 5$ ml during the second half of the menstrual cycle; during pregnancy $0 \cdot 1$ ml or even less was sufficient. Extraction was carried out by manual shaking once with 10 volumes and once with 5 vol. of petroleum ether (b.p. 40–60°C) in glassware of appropriate

size (25 × 180 mm round-bottom stoppered tubes when processing 4 ml of plasma; otherwise 12 × 140 mm tubes). Both petroleum ether extracts were combined and taken to dryness as indicated.

*Thin layer chromatography (t.l.c.).* The thin layer plates (10 × 20 cm) were divided into 6 lanes. The 4 central ones were 1·5 cm wide while the width of the 2 lateral lanes, used for progesterone standards, was 1 cm. Each lane was separated from its neighbor(s) by a scraped area 2 mm wide. A transversal furrow was drawn at 17 cm from the bottom in order to stop chromatography. The samples were transferred onto the activated plate in 3 successive 50 μl ethyl acetate fractions, as spots, at 2 cm from the bottom (pencil marks should be avoided!). The plates, each bearing 4 samples and 2 standards (5 μg progesterone) were then placed in a Shandon tank containing diethyl ether: benzene (9 : 5 v/v) as solvent. Ascending chromatography was allowed to proceed at room temperature.

Saturation of the tank before chromatography by solvent vapours was found to be superfluous. As many as 8 plates were developed using the same bath without recovery or final results being affected, provided solvent mixture was renewed daily.

After development of the chromatogram, the standards were visualized under ultraviolet light (254 nm) and circled. Two lines were drawn parallel to the solvent front, crossing the plate one cm above and one cm below the center of the standard spot respectively; care was taken not to spoil the silica layer, especially at the edges of the rectangles containing the material to be eluted. The standards were carefully scraped off the glass plate in order to eliminate progesterone-containing particles liable to contaminate the samples.

Areas containing the samples were then scraped off by means of a scalpel. For good recovery, scraping has to result in finely divided silica powder that is then transferred into 12 × 1 cm round bottom tubes. Elution was performed with 5 ml ethyl acetate, using a Vortex mixer the speed of which was progressively increased from '0' to '5'. Usually 15 s of this treatment allowed a good recovery that was not improved by increasing mixing time or speed. Thereafter the tubes were spun for 10 min at 3500 rev./min, the ethyl acetate extract was filtered over ethyl acetate-washed glasswool and evaporated to dryness in another set of similar tubes.

*LH-20 purification.* Mini-columns of Sephadex LH-20 were prepared as follows: the Pasteur pipettes were cut off just below the neck; a glasswool plug was inserted and compressed at the end of the cylindrical part (Fig. 1).

Each pipette was mounted in a special rack and washed with 3 ml chloroform: *n*-heptane : ethanol : water (200 : 200 : 1 : 50, by vol.)[6]. (The solvents were left in contact for at least 4 h: the aqueous phase was discarded just before use.) LH-20 and solvent mixture had been mixed for 15 min in a beaker prior to the preparation of the columns; usually, a score of columns was prepared at a time, which required about 6 g Sephadex and 140 ml solvent.

Sephadex sediments in a matter of seconds in these solvents; thus the mixture must be shaken by hand just before transfer onto the columns. For this purpose a Pasteur pipette, the extremity of which was broken so as to obtain an inner diameter of about 2 mm at the tip, was found convenient. The columns were considered ready when the Sephadex layer reached 3 cm in height. They were rinsed with 5 ml solvent before receiving the sample.

To each of the tubes containing the dry residue of the eluate from t.l.c. was
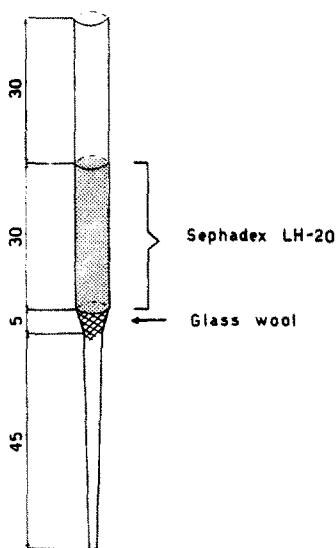
Fig. 1. Sephadex LH-20 mini-columns made from Pasteur pipettes. Dimensions are in mm.

added 0·5 ml of the solvent used on the column. The tubes were gently shaken to make sure that dissolution of the steroids was complete. and the solution was transferred onto the corresponding Sephadex column by means of a clean Pasteur pipette. The operation was repeated once with 0·5 ml, once with 1 ml solvent; the column was allowed to drain off before adding the following load of solvent. The 3 portions were collected together and mixed; an aliquot of 500 $\mu$l was removed from the 2 ml of eluate for determination of losses incurred up to this stage of the procedure while another aliquot of 1000 $\mu$l. meant for displacement analysis, was transferred onto a disposable culture tube in which it was taken to dryness.

*Displacement analysis:* A set of standards was prepared in duplicate containing 350 c.p.m. [1,2-$^3$H]progesterone and concentrations of 0, 0·5. 1, 2, 3. 4 and 250 ng of progesterone per 100 $\mu$l ethanol. respectively. They were taken to dryness in the usual way.

To tubes containing the dry residue of standard and samples. 1 ml of the CBG-solution was added and the tubes were shaken briefly (Vortex mixer. speed 4) before placing them in a water bath at 40°C with constant agitation for 10 min at minimal speed (Gallenkamp. Great Britain). They were transferred to an ice bath in which they were kept for 15 min before Florisil was added to each tube; this was immediately followed by mixing for 30 s with a Vortex mixer at maximum speed. For reproducibility it was found critical to allow the tube. during the shaking. to rotate around its longitudinal axis so that its fluid contents could swirl about the walls. The tube was then replaced in the ice bath; when mixing was completed for all tubes of the series. they were all centrifuged for 5 min at 3500 rev./min. making it possible to avoid, at the time of pipetting. contamination of supernatant with Florisil particles that otherwise adhere to the walls of the tube.

The tube was returned once more to the ice bath and 0·5 ml supernatant was transferred to a counting vial to which 10 ml of scintillation mixture were added; after 60 min at 4°C the vials were shaken and counted at a preset count of 20,000.

*Calculation.* A standard curve was plotted using '% bound' on the ordinate, and the amount of progesterone on the abscissa.

The term on the ordinate was calculated from:

$$\frac{S-N}{B-N} \times 100 \quad \text{where}$$

'*S*' corresponds to the c.p.m. in supernatant for each dose,
'*N*' stands for the mean c.p.m. in supernatant when total amount was 250 ng, and
'*B*' represents the mean c.p.m. recovered in supernatant when no unlabelled progesterone was added (0 dose).

The relationship between 'per cent bound' and dose (Fig. 2) makes it possible to establish progesterone concentration in samples. The actual amount of progesterone in each plasma sample is calculated by the following formula that takes into account overall losses:

$$\frac{X \times A \times 100}{2 \times C \times D} = \text{ng progesterone}/100 \text{ ml plasma, where}$$

'*X*' = amount of progesterone derived from visual inspection of standard curve,
'*A*' = c.p.m. labelled progesterone added to plasma sample,
'*C*' = c.p.m. recovered in 0·5 ml solvent after LH-20 column,
'*D*' = volume (in ml) of plasma assayed.

Three conditions had to be met before results for plasma were considered reliable:

(1) Final recovery greater than 50%.

(2) Sample amount (from reading on the standard curve) between 0·1 ng and 3·5 ng (Fig. 2).
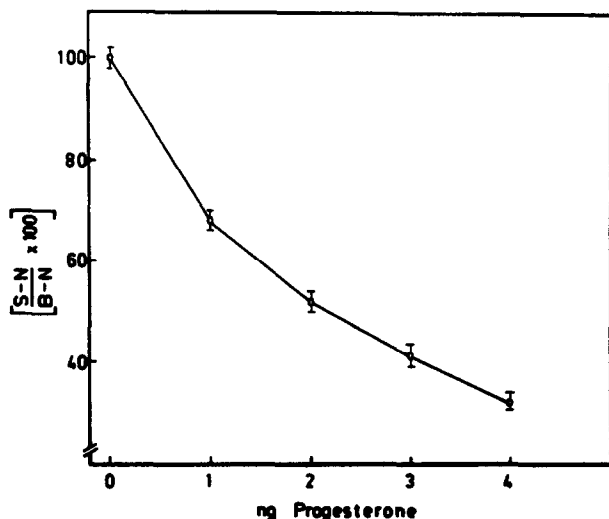
(3) Plasma duplicates within 15% of each other.



Fig. 2. Typical standard curve for progesterone determined with the binding system described. Open circles represent means of duplicates, and vertical bars stand for the range. For the meaning of the units used on the ordinate, see text.

COMMENTS

## 1. *The choice of solvents*

(a) *Extraction.* Petroleum ether has been widely recognized as an excellent solvent for the extraction of progesterone from biological fluids. Furthermore. it adds some specificity to the method, extracting only small fractions of most other plasma steroids [1,4,7].

In addition, plasma extracts with petroleum ether are by far cleaner than those with diethyl ether or ethyl acetate, which also extract almost quantitatively progesterone from plasma. Thus, choice of petroleum ether as the solvent is held to account for the observation that progesterone extracted from plasma behaves — in terms of $R_F$ — exactly like standard progesterone on t.l.c., allowing very good recoveries from the plate, i.e. $85 \pm 5\%$ (S.D.).

(b) *T.l.c. elution.* For this stage of the procedure ethyl acetate gave the best recovery, at the same time contributing the least interfering material. When using Merck precoated t.l.c. plates, methanol should be avoided because it elutes some binder used by the manufacturer and this substance interferes in protein-binding analysis (see below).

Interfering material also appeared in the eluates when deactivation (by water) was carried out prior to extraction of progesterone.

(c) *LH-20 Sephadex.* The LH-20 columns were resorted to in an attempt to reduce further the amount of non specific interfering material.

Three goals were sought: the eluate should be devoid of interfering substances, the amounts of solvents should be kept at a minimum, and elution should not be time-consuming.

From several systems tried, the proposed one turned out to be the most satisfactory.

Sephadex LH-20 is not completely inert regarding steroids: Fig. 3 shows the pattern of elution of progesterone and testosterone from our columns. Nevertheless, the main role of the column resided in it operating as a 'filter', retaining impurities coming from t.l.c., thereby diminishing appreciably the blank values.
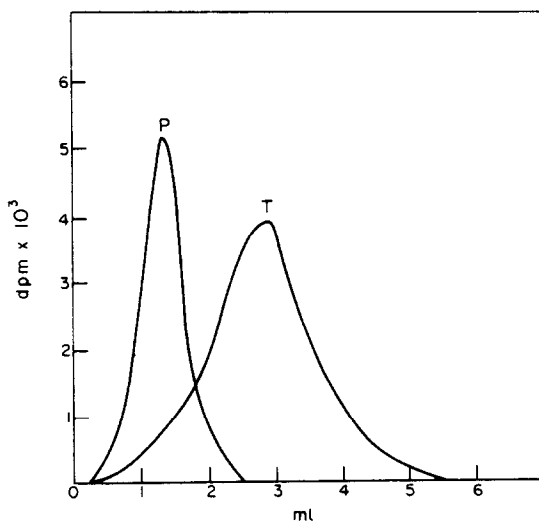


Fig. 3. Flution pattern of progesterone (P) and testosterone (T) from Sephadex LH-20 mini/ column. Solvent system: chloroform: *n*-heptane:ethanol:water (200:200:1:50. by vol. Aqueous phase was discarded just before use.)

On the other hand, some other solvents that *per se* do not contribute interfering material (i.e. the dry residues of which yield zero values in the assay) do so when used as eluants through LH-20 Sephadex. This was the case, most strikingly, with benzene–methanol mixtures.

## 2. *Influence of evaporation technique*

Traditionally, organic solvents are evaporated under a stream of nitrogen or, less frequently, under air or in a vacuum oven. In order to ascertain whether the choice of evaporation method influences the presence of interfering material in the final protein-binding assay, different sets of tubes were prepared, each containing the same volume of ethyl acetate (5 ml); after being taken to dryness at 70°C employing $N_2$ or air, or allowing evaporation to proceed in a laboratory oven (without vacuum), they were processed and read against a modified, more sensitive standard curve: 'blank' values averaged $0.1$ ng $\pm 0.03$ (S.D.) per sample after evaporation under a stream of air or nitrogen; when evaporation was carried out in an oven without circulation of gas, mean 'blank' was $0.06$ ng $\pm 0.016$ (S.D.) per sample. Substitution of ethyl acetate by methylene chloride or diethyl ether failed to modify appreciably these findings.

As will be discussed later, with the procedure described, values in this range are meaningless, i.e. they are not significantly different from zero. Therefore, evaporation under a stream of air was routinely adopted.

We also tested the influence of evaporation at 40°C and at 70°C; no differences were encountered and the higher temperature was selected for evaporation. Even after 2 h at 70°C under a stream of air, no denaturation had occurred since progesterone processed this way still migrated on t.l.c. quantitatively with the standard substance.

This temperature was obtained by means of a heating block, eliminating problems arising from use of water baths maintained at 70°C.

## 3. *The choice of precoated plates for t.l.c.*

The most important characteristic of the Merck plates is the resistance of the silica layer, which doesn't require the extreme care with which spotting should usually be performed, lest the plate be spoiled.

Furthermore, glass plates were preferred to non-rigid ones because there is no change resulting from the heat required for activation; therefore several plates can be run in a single tank with relative $R_F$ remaining very constant.

Washing t.l.c. plates prior to chromatography was investigated as to its repercussions on blank values. Methanol was again found to be detrimental, the blank being twice as high with methanol-washed plates $(0.940$ ng $\pm 0.827$ (S.D.)) as with unwashed ones $(0.585$ ng $\pm 0.177$ (S.D.)); worse yet, there were striking variations for a given plate, as appears from the very large S.D. mentioned above.

On the other hand, when the plate was washed with the solvent mixture used for chromatography of the plasma extracts, the blank averaged $0.424$ ng $\pm 0.123$ (S.D.) which is comparable to the value obtained with unwashed plates; this value dropped appreciably after the Sephadex LH-20 column, to $0.084$ ng $\pm 0.072$ (S.D.). As will be discussed below, values inferior to $0.1$ ng are without significance with the method described. Therefore, plates were used without washing.

When paper chromatography [3] was tried instead of t.l.c., recoveries were lower: $77 \pm 14\%$ (against $85 \pm 5\%$) because the rate of migration of plasma progesterone was not constant.

Furthermore, since blanks with paper were worse than with t.l.c., despite thorough washing of sheets (Whatman No. 540 'ash-less') with redistilled methanol for 48 h, and since paper chromatography was more time consuming and its recoveries more erratic, preference was given to t.l.c. for the assay of plasma progesterone.

## 4. *Usefulness of Sephadex LH-20*

That thin-layer chromatography leads to interference by unknown material upon subsequent quantitation of steroids by protein-binding, is a common experience.

It was reasoned that some kind of 'filter' could be suitable, hopefully hindering more or less specifically the migration of undesirable substances, thereby leading to an extract free of interfering material. Sephadex seemed a logical material to be tried. The choice of LH-20 was primarily conditioned by the fact that it withstands organic solvents.

In the meantime, Murphy[6] had submitted her first results obtained with Sephadex LH-20 and different solvent systems. Among those she had proposed, chloroform : *n*-heptane : ethanol : water (200 : 200 : 1 : to saturation, by vol.) gave us the best results in terms of blank values after passage through Sephadex LH-20 standard columns (20 × 1 cm); when benzene : methanol (1 to 5% methanol in benzene, by vol.) was resorted to, however, interfering material reappeared.

The next step consisted of reducing the size of the column; this way the steroid fraction could be collected in small volumes of eluate, so as to contribute lesser amounts of interfering material. Columns 3 cm high made of Sephadex LH-20 in Pasteur pipettes, were found appropriate.

Completeness of elution of progesterone in the conditions described was established by the fact that up to 50 ng of the steroid were recovered quantitatively. Furthermore, Sephadex LH-20 used for the experiment just mentioned was pooled, dried and re-suspended in a new batch of solvent; 10 columns were made with this pool and eluted. From each column an average of $0.085 \text{ ng} \pm 0.047$ (S.D.) was found: this is not significantly different from zero, for reasons discussed below.

Because of the quantitative elution of progesterone, Sephadex LH-20 could be reused, but its regeneration, being time-consuming, was not carried out routinely.

## 5. *Comments concerning the standard curve*

The addition to plasma of radioactive progesterone (as internal standard, so as to allow correction for procedural losses) raises a problem of interpretation of radioactivity measurement after competitive-binding assay; in tubes containing plasma sample residue there were on the average 350 c.p.m. radioactive progesterone (about 35% of the initially added radioactive progesterone) while the aliquot of CBG-solution contributed about 10,000 c.p.m. radioactive corticosterone.

Since binding constants for progesterone–CBG and corticosterone–CBG complexes are quite different and in view of the fact that the ratio between both favours by far corticosterone binding, it was predicted that the influence of the small amount of radioactive progesterone added would be negligible. This was assessed as follows: 2 sets of standards were processed, one containing only the

doses of unlabelled progesterone customarily used, while 350 c.p.m. of tritiated progesterone were added to each tube of the other series.

Table 1 presents results expressed in c.p.m.

Table 1. Establishment of standard curves in presence and absence of labelled progesterone

| Amount of progesterone (ng/tube) | Unlabelled progesterone | | | Labelled progesterone* | | |
|---|---|---|---|---|---|---|
| | Mean | S.D. | S.E.M. | Mean | S.D. | S.E.M |
| 0 | 6638 | 111 | 50 | 6958 | 236 | 96 |
| 1 | 5134 | 201 | 82 | 5040 | 220 | 90 |
| 2 | 3974 | 135 | 61 | 3829 | 53 | 21 |
| 3 | 3348 | 73 | 30 | 3466 | 164 | 67 |
| 4 | 2943 | 77 | 31 | 3157 | 36 | 15 |
| 250 | 1332 | 107 | 44 | 1342 | 58 | 29 |

At no concentrations were differences statistically significant.
*350 c.p.m.. corresponding to 0·012 ng progesterone.
For each point $n = 6$.

From a statistical point of view, there is no difference between both situations. Nevertheless, the standard curve (addition of radioactive progesterone) is steeper than the control one in its initial portion; there is no obvious explanation for this fact. At any rate the proposed standard curve was adopted since both samples and standards were handled in a comparable way.

For practical purposes, subtraction of 'non specific binding' yields a sharper standard calibration curve[8].

The linearization of the standard curve as proposed by Rodbard et al.[9] has unquestionable theoretical advantages. but a computer should be used for proper treatment[10]. This is not indispensable: visual inspection of the curvilinear relationship obtained by plotting the 'per cent bound' versus the dose is satisfactory and a technician does not commit errors provided that the scales are reasonably chosen.

A word of caution should be said about figures close to zero readings: as discussed in the next section it is not justified to consider values smaller than 0·1 ng as significantly different from zero.

As pointed out by Lipsett et al.[8], separation of free from bound moieties of the steroid represents a critical step in protein-binding assays. An incomplete separation will necessarily lead to unreliable standard curves.

Two approaches could be proposed to achieve proper separation: adsorption of free steroid on Sephadex G-25 columns, charcoal–dextran suspension or Florisil, or adsorption of the CBG–corticosterone complex: attempts to this end were made with DEAE-Sephadex, but standard curves were no better than those obtained by counting the bound steroid as proposed.

At this stage, Florisil has been retained because of ease of manipulation and reproducibility of results.

Dog CBG yielded curves less sensitive than those obtained with human CBG, as previously reported[7]. Moreover, at least in our hands, human CBG solutions were more stable and gave better duplicates than those obtained with dog CBG.

## ASSESSMENT OF THE METHOD

### 1. *Precision and accuracy*

For assessment of the precision of the present procedure. repeated assays of plasma samples containing known amounts of progesterone were processed at random; thus, three pools of plasma from normal adult males and one pool from normal females at mid cycle were prepared, and progesterone was added to plasma in amounts ranging from 0·8 to 20·3 ng/ml.

Results are summarized in Table 2, in which 'mean' reflects accuracy and 'S.D.' precision.

It has been claimed that duplicates yield closer data when binding approximates 50%, resulting in improved precision[8]; therefore the bound fraction is given in the table. It appears that precision was good, as pointed out by coefficients of variation, irrespective of the final concentration of progesterone in plasma and of the fraction bound.

The reason for the relative lack of influence of per cent binding on precision is thought to lie in personal variations in sample handling that exceed variations arising from departure from optimal reading conditions (50% binding of assayed material).

Accuracy was established (Table 3) by processing different volumes of a given plasma pool (Pool M III, from Table 2).

Conditions were selected, in terms of amounts of progesterone dealt with. in order to get readings on the standard curve that would correspond to those usually encountered through a normal menstrual cycle taking into account the volume of the extracted sample. It can be seen that the size of samples. hence the amount of hormone to be measured. fails to modify appreciably the coefficient of variation. It should be emphasized that these assays were carried out at random*. by two

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*(Except for Pool M V in Table 2 in which progesterone levels exceeded the useful range of the standard curve when the usual aliquot was submitted to the protein-binding assay.)

Table 2. Progesterone measurements: precision

| | Plasma progesterone (ng/ml) | | | | | | | |
| | | | Measured | | | | | % binding of the mean on the standard |
| Pool | Endogenous | Added | Mean | S.D. | S.E.M. | $n$ | $C$ | curve |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| M I* | 0·25 | 0·80 | 0·84 | 0·15 | 0·05 | 10 | 17·6 | 78 |
| F I | 2·76 | — | 2·76 | 0·16 | 0·08 | 4 | 6·0 | 80 |
| M II | 0·19 | 0·85 | 1·06 | 0·09 | 0·03 | 8 | 9·0 | 74 |
| M III | 0·40 | 3·60 | 3·66 | 0·38 | 0·07 | 5 | 10·4 | 70 |
| M IV | 0·40 | 11·30 | 11·57 | 1·60 | 0·51 | 10 | 13·8 | 56 |
| M V | 0·40 | 20·30 | 20·87 | 2·28 | 0·72 | 10 | 10·9 | 46(1) |

*Plasma samples assayed were 0·5 ml. except for Pools M I and M II (2 ml): M stands for plasma from males; F. from females at mid-cycle.

(1) Half of the amount usually processed was submitted to binding assay.

$n$: number of assays, $C$, coefficient of variation. S.D.: standard deviation. S.E.M.: standard error of the mean.

Table 3. Progesterone measurements: accuracy

| Plasma vol. assayed | Progesterone present in sample (ng) | | | | | Plasma Progesterone Concentration ng/100 ml |
|---|---|---|---|---|---|---|
| | Mean | S.D. | S.E.M. | $n$ | $C$ | |
| 0·5 ml | 1·69 | 0·19 | 0·08 | 5 | 11 | 338 |
| 1 ml | 3·87 | 0·15 | 0·07 | 5 | 4 | 387 |
| 2 ml | 7·48 | 0·77 | 0·34 | 5 | 10 | 374 |

Plasma used was from pool M III (Table 2). $n$: number of duplicates, $C$: coefficient of variation.

technicians and at different times. Thus, coefficients of variation given here reflect interassay differences rather than intraassay variations.

This is illustrated by data obtained with Pools M I and M II, the latter having been processed by one technician in a single run — hence, in all likelihood, the improved figures.

Therefore, it is advisable for samples collected through a given menstrual cycle to be processed as a series[17].

## 2. *Sensitivity*

If, as defined by Lipsett *et al.*[8], sensitivity is determined by the 95% confidence limits of the zero dose, for the assay as described here sensitivity is $0 \pm 0·09$ ng. Thus, values smaller than 0·1 ng are meaningless. When this occurred with biological material, larger samples were processed so as to exceed the threshold of the procedure.

Since recovery of radioactive progesterone, up to the binding step, averaged 70% and since half of the material was used for the binding assay, as little as 0·28 ng progesterone could be significantly distinguished from zero..

Thus, at least theoretically, the minimum concentration of plasma progesterone detectable with the present procedure is 7 ng/100 ml if 4 ml plasma are extracted. The limit becomes 56 ng/100 ml when 0·5 ml is processed.

## 3. *Specificity*

In the present case, specificity should be defined as the property of the method to measure progesterone exclusively.

Different factors contribute to this aim, with the procedure described: (a) choice of the solvent for extraction (see above); (b) thin-layer chromatography that allows isolation of progesterone ($R_F$ 0·59 ± 0·009) from 17α-hydroxyprogesterone ($R_F$ 0·31 ± 0·003) and 20α-hydroxy-4-pregnen-3-one ($R_F$ 0·31 ± 0·005); these steroids must be eliminated because they are also extracted to a sizable extent by petroleum ether (31% and 60%, respectively); (c) binding characteristics of CBG·[2.7.11]; (d) constancy with which blank values are included in the range not distinguishable from zero: with the method as described, processing of 2 ml or less of charcoal-treated plasma — allegedly steroid-free at this stage — yielded a zero value for progesterone by the criteria discussed.

With 4 ml of plasma from an ovariectomized women treated for 5 yr with pharmacological doses of corticosteroids a zero value was also read on the standard curve.

The best argument for specificity of the procedure is probably provided by results obtained in some well-documented situations. In 12 normal males. aged 20–30. mean plasma progesterone was $0.24 \pm 0.18$ (S.D.) ng/ml. a figure comparing favourably with those obtained by double dilution[12] or gas–liquid chromatography techniques[13]. In 5 healthy young women. plasma progesterone was determined daily through the menstrual cycle; Fig. 4 illustrates the data organized about the mid-cycle LH* surge taken as a chronological index.
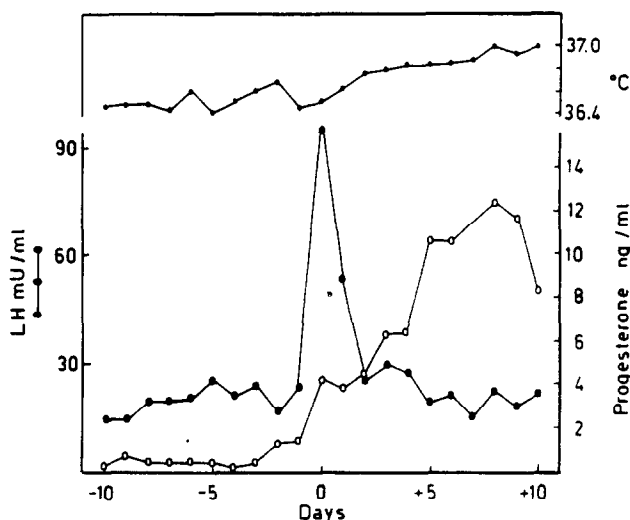


Fig. 4. A composite graph of 5 normal menstrual cycles. centered on mid-cycle plasma LH peak (day 0). For comments see text.

Mean levels were $0.7$ ng/ml at most during the early part of the follicular phase: a small rise occurred coincident with the LH peak. followed by the expected rise typical of the luteal phase of the cycle. with maximal plasma progesterone by the 7th or 8th day after the LH peak.

These results are similar to those reported by other investigators[1.2.15.16].

In sum, a sensitive and precise technique has been developed for measurement of progesterone in amounts such as encountered in human plasma. Sephadex LH-20 proved a most useful means aimed at overcoming problems raised by high. variable blank values that interfered whenever chromatography was resorted to for proper isolation of progesterone prior to protein-binding assay.

Specificity and accuracy are likely to account for small yet reproducible variations of plasma progesterone such as observed in the periovulatory period (Fig. 4). This event, already alluded to by other investigators, deserves attention only in so far as progress in methodology makes it possible to put proper reliance on discrete changes in plasma progesterone concentrations.

*LH is expressed in mUHMG/ml plasma (Int. Standard of H.C.G.). It was kindly measured by Dr. K. Thomas[14]. Dépt. de Gynécologie. U.C.L.

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