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SALIVARY PROGESTERONE: RELATION TO TOTAL AND NON-PROTEIN-BOUND BLOOD LEVELS

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Summary—The daily amounts of salivary progesterone have been determined over the complete menstrual cycle for 9 normal women. The level of progesterone during the follicular phase was about 150 pmol/l and increased significantly to about 350 pmol/l during the luteal phase of the menstrual cycle. The amounts of salivary progesterone were significantly correlated with those in blood ($P < 0.001$) in paired saliva–blood specimens taken from 96 women. Although these volunteers comprised patients with benign and malignant breast disease and normal unaffected women, the relationship between salivary and blood progesterone was similar for all groups. The concentration of non-protein-bound progesterone was determined using equilibrium dialysis. To correct for serum dilution the linear relationship between the percentage of progesterone bound and the reciprocal of serum dilution has been exploited. The values of non-protein-bound progesterone obtained were significantly and linearly correlated with levels in saliva ($r = 0.75$, $P < 0.001$, d.f. = 34) although the amount of free progesterone in blood was about five times that found in saliva.

INTRODUCTION

There are a number of practical advantages in measuring progesterone in saliva rather than in blood for the assessment of endocrine function [1]. The technique is non-invasive and suitable for the collection of frequent samples. It has also been claimed that salivary steroid levels provide an accurate estimate of plasma levels and that they also reflect the amount of steroid not bound to carrier proteins in the blood [2], since it is widely believed that only the free fraction of steroid hormones is biologically available [3] it follows that measurement of salivary progesterone might be more informative than conventional determinations using blood in assessing luteal phase function [4, 5].

The evidence that salivary assays have all the advantages listed above is exiguous and we have, therefore, examined the relationship between total and non-protein-bound progesterone and salivary progesterone.

EXPERIMENTAL

Reagents

[1,2,6,7-^{3}H]progesterone (80 Ci/mmol) was purchased from Amersham International plc, Bucks, England. The tritiated progesterone was purified on a column (10 × 1 cm) of Sephadex LH20 using a mixture of hexane, toluene and methyl alcohol (8:1:1, v/v) before use. The characteristics of the monoclonal antibody (11P27) used for radioimmunoassay have already been described [6]. Radioimmunoassay buffer was phosphate-buffered physiological saline (pH 7.4) containing gelatine and sodium azide each at a concentration of 0.1% (w/v).

Saliva and blood

Saliva samples were collected by direct salivation and samples stored at −20°C. In the case of matched saliva and blood specimens the saliva was collected within 15 min of venepuncture. Blood specimens were allowed to clot at room temperature for 1 h and the resulting sera stored at −20°C.

Subjects

Nine normal women aged 21–40, with a history of regular cycles provided daily saliva samples for a complete menstrual cycle. Matched samples of saliva and blood were taken at random times in the cycle from an additional 15 normal women, from 22 patients with breast cancer before and after treatment and from 37 women with benign breast disease.

Measurement of salivary progesterone

Saliva which had been stored at −20°C was thawed and particulate matter removed by centrifugation. The saliva (400 μl) was extracted with 3 ml of isopentane, the mixture was centrifuged then frozen and the organic phase was decanted and the isopentane removed by evaporation. Ethyl alcohol (10 μl) was added to the dried extract followed by radioactive progesterone (5000 dpm) and antibody to a final volume of 200 μl. The assay tubes were incubated at 37°C for 45 min and at 4°C for 45 min after which 200 μl of charcoal (0.5 mg) coated with dextran (DCC) (0.05 mg) was added. The assay tubes were mixed and kept at 4°C for 30 min before

centrifugation. The supernatant from the tubes was decanted into counting vials and radioactivity determined. For recovery purposes saliva containing a known amount of tritiated progesterone (5000 dpm) was processed as described above, except the addition of DCC was omitted, and recovered tritium determined. The mean recovery was 85% with a coefficient of variation of 5%. Quality control salivas from a low and high titre pool were included in each batch of samples and the mean titres for 18 assays were 146 pmol/l and 390 pmol/l, with coefficients of variation of 15 and 10%, respectively.

The lowest amount measurable with a 95% certainty was 6 pg/tube which in this assay is equivalent to 54 pmol/l.

Saliva samples taken from women during the luteal or follicular phases of the menstrual cycle had no detectable progesterone after treatment with charcoal.

Accuracy of the assay was assessed by the measurement of progesterone after the addition of known amounts of steroid. The results in Table 1 showed that the results of these experiments were satisfactory.

The specificity of the method was deemed satisfactory since assay of various amounts of saliva extract (from both luteal and follicular phases of the cycle) gave inhibition curves which were parallel to those of standard progesterone and the monoclonal antibody is highly specific [6]. Furthermore, the results obtained are in agreement with those in the literature (see the Discussion).

Measurement of blood progesterone

The assay of serum progesterone was essentially of that described previously [7] except for the use of the monoclonal antibody 11P27 [6]. Under the conditions of this assay the least amount detectable with 95% certainty was 10 pg/tube or 3 nmole/l.

Determination of progesterone binding in serum

Solutions containing 50, 25, 17.25 and 12.5% of serum in physiological saline were prepared. These solutions (0.3 ml) were dialysed at 37°C in physiological saline containing tritiated progesterone (5000 cpm/ml) in $\frac{1}{4}''$ Visking casing. The four sacs were equilibrated in one glass Universal tube containing 14 ml of radioactive progesterone in saline with gentle shaking in a water bath at 37°C for 18 h.

Table 1. Assessment of accuracy

Progesterone added (pmol/l)	Follicular phase		Luteal phase	
	Measured (pmol/l)	Difference	Measured (pmol/l)	Difference
0	110 ± 9[a]		290 ± 21[a]	
50	163 ± 14	53	341 ± 32	51
100	214 ± 18	104	396 ± 35	106
200	323 ± 22	213	478 ± 43	188
300	395 ± 31	285	612 ± 51	322
400	533 ± 42	423	703 ± 65	413

[a]Mean ± SD, with $n = 6$.

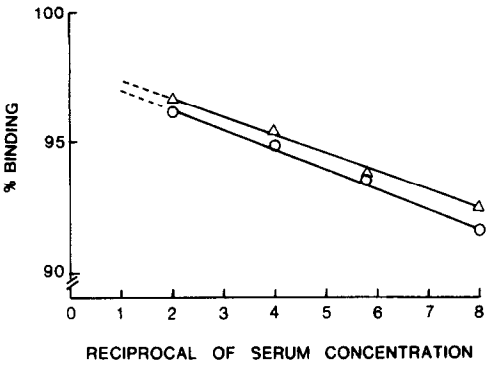

Fig. 1. Binding of progesterone to serum at various dilutions. The percentage of radioactive progesterone bound to two different serum samples, as determined by equilibrium dialysis, is plotted against the reciprocals of the sera concentrations. These reciprocals have been calculated using the equation $100/x$, where x is the percentage of serum. The broken lines show extrapolation to 100% serum concentration.

Control experiments showed that this time was sufficient for equilibrium to be reached. After equilibration the sacs were removed from the dialysis vessels, blotted and the contents (2 × 0.1 ml) counted for radioactivity, together with four aliquots of the dialysate (0.2 ml). The percentage of radioactive progesterone bound to the various diluted serum solutions was calculated using absolute counts (dpm).

To determine the amount of progesterone bound to undiluted serum the linear correlation equation was calculated using percentage radioactivity bound (y) against the reciprocal of the serum dilution (x). Thus the 50, 25, 17.25 and 12.5% dilutions become 2, 4, 5.8 and 8, respectively. The percentage of progesterone bound to undiluted serum (100%) can be obtained by extrapolation of the linear regression equation to a point where $x = 1$. Two examples are shown in Fig. 1. The regression coefficient was in excess of 0.99 for both sera.

Sera were assayed in batches of 20 together with a quality control. For 7 such batches the mean regression coefficient was 0.994 with a coefficient of variation of 1%. The mean percentage for unbound progesterone for the quality controls was 3.05% with coefficient of variation 9%. The mean of all the sera was 2.98% (±0.42%, SD).

The percentage of bound progesterone remained constant over the menstrual cycle; the mean values being 3.01, 2.98 and 2.98% for days 0–10, 11–20 and 21–30 of the cycle, respectively.

Five samples of pregnancy sera gave mean of 2.09% (±0.28, SD) for unbound progesterone.

RESULTS

Salivary progesterone and the menstrual cycle

The mean amounts of salivary progesterone ranged from 100 to 150 pmol/l during the follicular phase and increase significantly to about 350 pmol/l in the

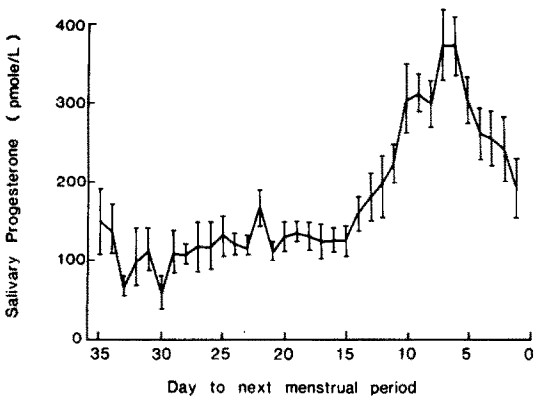

Fig. 2. Daily salivary progesterone levels over the menstrual cycle based on the day to next menstrual period. The mean daily salivary progesterone for 9 normal women are plotted against day to next menstrual period. The scatter bars represent standard errors of the means.

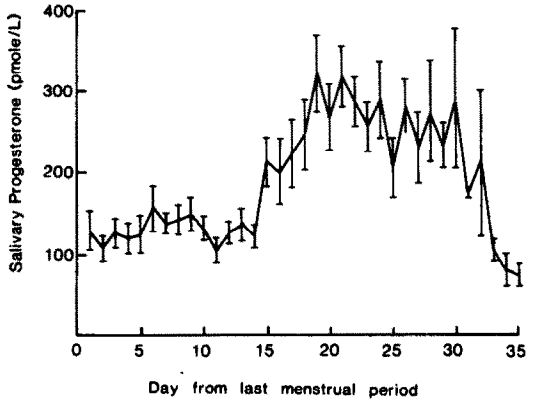

Fig. 3. Daily salivary progesterone levels over the menstrual cycle based on the day from last menstrual period. The mean daily salivary progesterone for 9 normal women are plotted against day from last menstrual period. The scatter bars represent standard errors of the means.

luteal phase of the menstrual cycle (Fig. 2). The peak of progesterone is considerably wider if values are plotted using the last menstrual period rather than the next menstrual period (Fig. 3). This is due to the between-person variation in cycle length which in these volunteers ranged from 26 to 35 days.

Comparison of salivary and total serum progesterone

The relation between blood and salivary progesterone for the 96 women from whom random blood samples were taken is shown in Fig. 4. The correlation was significant ($r = 0.53$, d.f. $= 94$, $P < 0.001$) and the coefficient of determination (r^2) was 28%. The relationship between salivary and blood progesterone levels was similar for all women irrespective of clinical status. There were 7 subjects whose blood progesterone was less than 10 nmol/l, indicative of follicular phase, but where salivary progesterone was in excess of 300 pmol/l. Two of these subjects had levels in excess of 700 pmol/l. Further matched blood and saliva samples from some of these subjects did not show this anomalous behaviour. Of these 7 anomalous specimens there was too little saliva left to analyse the impurity by GLC. However, the saliva of these samples were pooled and extracted with hexane. The extract did not show parallel inhibition to an authentic progesterone standard although extracts from a pooled saliva of other samples taken during the follicular phase did.

Comparison of the amounts of progesterone not bound to blood protein with those in saliva

All results which were below the lower limit of sensitivity for either the saliva or blood assays were excluded and this yielded 36 matched pairs which are shown in Fig. 5. The linear correlation coefficient of salivary and total blood progesterone was 0.78 ($P < 0.001$).

The mean percentage of progesterone bound was 2.98% (± 0.42, SD) in these blood specimens. The amount of free progesterone was computed using the total progesterone concentration and percentage binding for each blood specimen. Comparison of progesterone levels in saliva and the amount of unbound progesterone showed a high degree of linear correlation ($r = 0.75$, $P < 0.001$) with a slope of 0.14 (Fig. 6).

DISCUSSION

This study, in agreement with others, shows that the levels of salivary progesterone increase during the

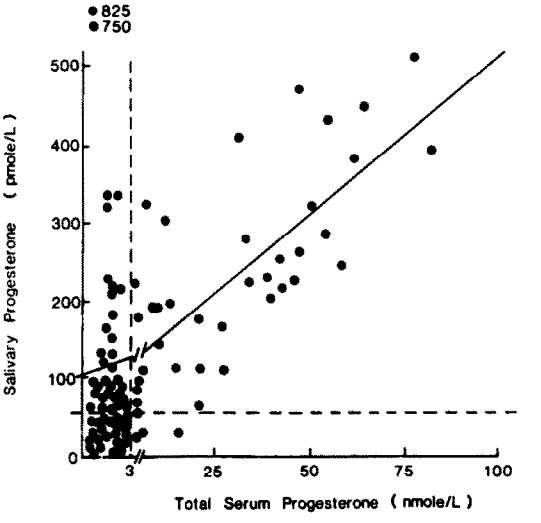

Fig. 4. Relationship between salivary and total blood progesterone. The equation of the linear regression of progesterone levels in saliva and blood in 96 women is $y = 4.08x + 103$, where y is the amount of salivary progesterone (pmol/l) and x is the total amount of blood progesterone (nmol/l). The regression coefficient is 0.53 ($P < 0.001$). The broken lines represent the limits of sensitivity for the salivary and blood progesterone assays. For these paired samples blood was taken immediately after saliva collection. For convenience the horizontal axis is discontinuous.

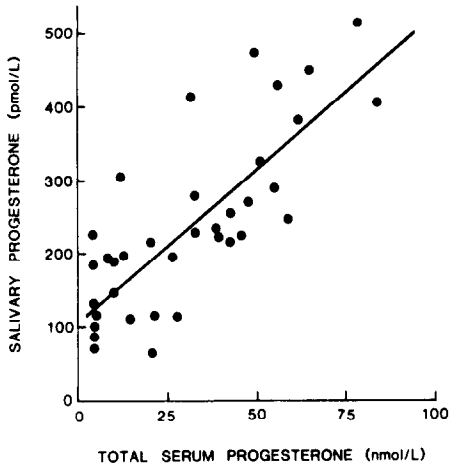

Fig. 5. Relationship between salivary and total blood progesterone. The data are the same as that shown in Fig. 4, except progesterone values below the sensitivity of either the salivary or blood assays have been omitted. The equation of the linear regression of the 36 women is $y = 4.19x + 105$, where y is the amount of salivary progesterone (pmol/l) and x is the total amount of blood progesterone (nmol/l). The regression coefficient is 0.78 ($P < 0.001$).

luteal phase of the menstrual cycle. This rise is most marked if plotted using the date of the next, rather than the last, menstrual period. The peak level of salivary progesterone is about 350 pmol/l and therefore, in accord with most authors (Table 2).

There is a significant linear relationship between the amounts of progesterone in blood and in saliva. A close linear correlation has been reported by Walker *et al.*[13]. However, what was noticeable in the present data was that a few of the saliva samples

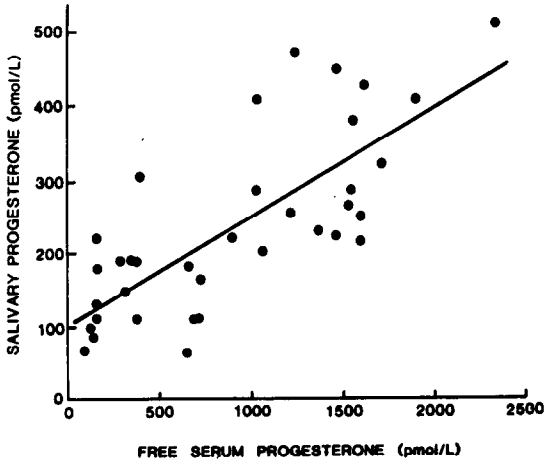

Fig. 6. Relationship between salivary and free blood progesterone. The amount of free blood progesterone has been computed from the total blood levels of the steroid and the percentage of non-protein-bound progesterone for each blood specimen. The equation of the linear regression of the 36 women is $y = 0.14x + 106$, where y is the amount of salivary progesterone (pmol/l) and x is the concentration of free progesterone in blood (pmol/l). The regression coefficient is 0.75 ($P < 0.001$).

Table 2. Mean luteal phase levels of salivary progesterone

Author	Titre (pmol/l)
Luisi *et al.*[8]	3000–4000
Shah and Swift[9]	900
Sorgo *et al.*[10]	500–600
Choe *et al.*[11]	475
Jeffcoate[12]	400–500
Walker *et al.*[13]	300–400
Cedard *et al.*[14]	300–400
Tallon *et al.*[15]	300
Present study	300–350

had appreciable amounts of immunoreactive progesterone even though the corresponding blood levels of hormone and the day of the cycle indicated that these volunteers were in the follicular phase of the cycle. The source of the progesterone is unlikely to originate from blood contamination since some of these saliva samples would need to have up to 25% of blood present. In spite of the rigorous efforts made to ensure that the assay was specific the evidence presented in this paper suggested that these occasional high levels are due to the presence of immunoreactive impurities. Even though volunteers were asked to rinse their mouths with water 5 min before collection the most likely source of the impurity is from food debris. To support this was the fact that a subsequent saliva sample from such a volunteer did not have a high progesterone level.

A difficulty encountered when measuring steroid binding to serum using equilibrium dialysis is correcting for the dilution of serum. For example, in the report of Smith *et al.*[16], in which they compared the concentrations of testosterone in saliva with the unbound steroid in serum they applied a correction factor although it is not clear how they obtained the value for this factor. In the present method we have exploited the linear relationship between the reciprocal of serum concentration and percentage bound. This straight-line association was apparent up to a serum dilution of 6% and may be generally applicable since a similar relationship was observed for cortisol. The method appears to be satisfactory for estimating the amount of unbound steroid in undiluted sera since the present average results of 2.98% agree with 2.54% by centrifugal ultrafiltration [17], 2.38% by a solid-phase method [18] and 3.2% by multiple equilibrium dialysis [19]. In addition the 2.09% of progesterone found to be unbound in pregnancy sera is in accord with the range 1.76–2.77% reported by Anderson *et al.*[20] using steady-state gel filtration.

From the present results it seems unlikely that the amount of progesterone in saliva is equal to the concentration of unbound steroid in the blood. Although there is a high degree of linear correlation between the parameters, the slope of the line is only 0.14. To attain a situation where the amount of free progesterone was directly equivalent to that in saliva would require the percentage of unbound pro-

gesterone to be of the order of 0.5% a value far below that found even in pregnancy [20]

Other evidence that the levels of salivary steroids are equal to the amounts of free or biologically available steroids is equivocal. Umeda *et al.*[21] found that the concentration of cortisol in saliva was 30% less than that of free cortisol in serum and prompted them to suggest that the passage of steroid from the blood stream to parotid fluid involved more than simple diffusion. This could be the result of conversion of cortisol to cortisone by salivary gland [22]. Similarly Baxendale and James[23] reported that in women the amount of salivary testosterone was nearly three times that found for free testosterone in blood and both Smith *et al.*[16] and James and Baxendale[24] found the slope of the linear regression of these two parameters to be 20% less than unity in women. It has been suggested that this discrepancy could be due to metabolism of testosterone by salivary gland or to the presence of sex hormone binding globulin (SHBG) in saliva. However, Wang *et al.*[25] have reported the absence of SHBG in saliva.

The results of this study show that there is a significant correlation between the amounts of salivary progesterone and free progesterone in blood although these levels were not directly equivalent. There was an equally significant correlation between salivary and total blood progesterone. This study also confirms the usefulness of salivary assays for the monitoring of ovarian function over the menstrual cycle.

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