Salivary cortisol: a better measure of adrenal cortical function than serum cortisol

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SUMMARY Salivary cortisol concentration was found to be directly proportional to the serum unbound cortisol concentration both in normal men and women and in women with elevated cortisol-binding globulin (CBG). The correlation was excellent in dynamic tests of adrenal function (dexamethasone suppression, ACTH stimulation), in normals and patients with adrenal insufficiency, in tests of circadian variation and randomly collected samples. Women in the third trimester of normal pregnancy exhibited elevated salivary cortisol throughout the day. The relationship between salivary and serum total cortisol concentration was markedly non-linear with a more rapid increase in salivary concentration once the serum CBG was saturated. The rate of equilibrium of cortisol between blood and saliva was very fast, being much less than 5 minutes. These data, combined with a simple, stress-free, non-invasive collection procedure, lead us to suggest that salivary cortisol is a more appropriate measure for the clinical assessment of adrenocortical function than is serum cortisol.

The measurement of steroid concentrations in saliva has a number of potential advantages over the more conventionally used total serum concentrations. These advantages include a stress-free and non-invasive collection procedure and the measurement of a parameter which is believed to reflect the biologically active, serum unbound steroid concentration. Accordingly, the potential of measurements of the salivary concentrations of a number of steroids have recently been investigated.1-7

The potential value of a measurement of corticosteroids in saliva was recognised as early as 19598,9 but the lack of sensitive and specific assays hampered the development of the technique. More recently, it has been shown that cortisol may be measured in small volumes of saliva using radioimmunoassay.10 Also Umeda et al.11 have shown that the salivary cortisol concentration correlates well with the serum free cortisol concentration, although their data are very dependent upon supraphysiological cortisol levels obtained by intravenous injection of cortisol.

We have (1) investigated the relationship between salivary cortisol concentration, serum total cortisol concentration, and serum unbound cortisol concentration at normal physiological levels; (2) assessed the value of measurements of salivary cortisol concentration in several commonly used tests of pituitary-adrenal cortical function, ie, dexamethasone suppression, adrenocorticotrophin (ACTH) stimulation, and circadian variation; (3) examined the circadian variation of salivary cortisol in women in the third trimester of normal pregnancy; and (4) examined the rate of appearance of cortisol in saliva.

Materials and methods

SUBJECTS

Circadian variation The circadian rhythm of salivary cortisol was examined in seven female and seven male healthy adults (age range 24–32 years). Each subject collected a saliva sample every hour for one day throughout their normal waking hours. On another day the same groups provided matched serum and saliva samples at 0900 and 1700. The circadian rhythm of salivary cortisol in the third trimester of normal pregnancy was examined in 13 sets of samples from 10 women (age range 22–35 years) with uncomplicated pregnancies who delivered healthy single babies at 38–42 weeks' gestation.

Dexamethasone suppression/ACTH stimulation Six male and five female healthy young adults received dexamethasone (1 mg orally) at 2200, and samples of serum and saliva were obtained at 0900 the next day.
Each subject then received tetracosactrin (Synacthen, 250 µg im at 1500), and time-matched samples of serum and saliva were collected after 0, 30, 60, and 120 min. Further saliva samples were collected at 180, 300, and 420 min. Several patients being investigated for adrenal insufficiency also collected time-matched serum and saliva samples during an ACTH stimulation test.

**ACTH infusion** Six normal healthy male volunteers aged 18-31 years provided time-matched saliva and serum samples for this study. Each subject received dexamethasone (1 mg orally) the night before the study at 2300 and the next morning was given an infusion of tetracosactrin (Synacthen) starting at 0-2 µg/15 min with a stepwise doubling (approximately) every 15 min to reach a peak of 80 µg/15 min. The total dose administered was 160 µg over a 2-hour period. Time-matched samples of serum and saliva were obtained at various times throughout the study.

**Appearance rate** The rate of appearance of cortisol in the saliva was assessed in normal young adults (2 male, 3 female, age range 27–31) who were each given an intravenous injection of 5 mg cortisol sodium succinate into the antecubital vein of the right arm. Time-matched samples of saliva and serum (from antecubital vein of left arm) were obtained from each subject at -5, 0, 5, 10 and 20 min.

**Collection of samples** Collection and storage of saliva and serum samples was as previously reported.

**Materials**
Cortisol, radioimmunoassay (RIA) standard, was purchased from Steraloids Inc, Wilton, NH, USA and cortisol antiserum (No. CO01) from Steranti Research Ltd, St. Albans, Herts, England. Tritium-labelled cortisol (1, 2, 6, 7 $^3$H(n)) was obtained from New England Nuclear, Boston, Mass, USA and was used for RIA without further purification. The tritium-labelled cortisol used for the determination of the serum free-cortisol fraction was purified by column chromatography on silica gel (Kieselgel 60, 230–400 mesh, Merck, Dermstadt, FRG) with benzene/ethyl acetate (9/1) as eluate. Assay buffer, methylcellulose-coated charcoal, and liquid scintillant were as previously reported.

Cortisol sodium succinate (hydrocortisone sodium succinate; Efcortelan 100 mg BP for injection) was purchased from Glaxo Australia Pty Ltd, Boronia, 3155, Australia. Dexamethasone (0-5 mg tablets) was purchased from Protea Pharmaceuticals Pty Ltd, Glebe, 2037, Australia. Synthetic 1–24 adrenocorticotrophic hormone (ACTH) (Synacthen, tetracosactrin BP) was purchased from Ciba Pharmaceuticals, Lane Cove, 2066, Australia.

Unflavoured chewing gum (non-tack, base material, Sydney) was supplied by the Wrigley Co Pty Ltd, Asquith, 2078, Australia.

**Methods**
Cortisol was determined by RIA using a method similar to that described by Carr and co-workers. Briefly, saliva (50 µl) or diluted serum was heated to 70°C for 10 min; antiserum and tritium-labelled cortisol were added, and the mixture was incubated overnight at 4°C. Bound and free cortisol were separated with methyl cellulose coated charcoal, followed by centrifugation, and the activity of the supernatant was determined.

Separation of free from protein-bound cortisol in serum was effected by centrifugal ultrafiltration using a micropartition system (MPS-1, Amicon Corporation, Scientific Systems Division, Mass, USA). The percentage of unbound cortisol in serum was measured by centrifugal ultrafiltration as previously described.

**Results**

**Analytical Variables**
The antiserum, which was raised in rabbits against the antigen, cortisol-3-O-(carboxymethyl) oxime-human serum albumin, exhibits a high specificity for cortisol. The only steroids with more than negligible cross reaction were cortisone (0.3%), 11-deoxycortisol (9%), and 21-deoxycortisol (22%). This degree of cross reaction is unlikely to cause a significant bias in the assay.

The sensitivity of the assay was 5 pg/assay tube. The assay precision, assessed by intra- and inter-assay coefficients of variation on several pools of serum and saliva (Table 1), was typical of steroid

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Precision of the cortisol assays</th>
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</thead>
<tbody>
<tr>
<td><strong>Assay</strong></td>
<td><strong>x (nmol/l)</strong></td>
</tr>
<tr>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>Intra assay</td>
<td>169</td>
</tr>
<tr>
<td>intra assay</td>
<td>398</td>
</tr>
<tr>
<td>Inter assay</td>
<td>1074</td>
</tr>
<tr>
<td>Inter assay</td>
<td>168</td>
</tr>
<tr>
<td>Inter assay</td>
<td>397</td>
</tr>
<tr>
<td>Inter assay</td>
<td>1028</td>
</tr>
<tr>
<td>Saliva</td>
<td></td>
</tr>
<tr>
<td>Intra assay</td>
<td>11.2</td>
</tr>
<tr>
<td>Intra assay</td>
<td>1.43</td>
</tr>
<tr>
<td>Inter assay</td>
<td>11.3</td>
</tr>
<tr>
<td>Inter assay</td>
<td>1.41</td>
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</table>
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RIA. The precision of the ultrafiltration method for measuring the percent of unbound cortisol in serum samples was typically 2.2% (mean CV based on several measurements from several serum pools).

A number of samples of serum and saliva with high cortisol levels were assayed in various dilutions. The measured cortisol concentration was found to be independent of the volume assayed.

The use of a salivation stimulus such as unflavoured chewing gum resulted in easier and faster collection of saliva samples and had no effect upon saliva cortisol concentration, since sequential saliva samples collected with or without chewing gum had the same cortisol concentration.

RATE OF APPEARANCE OF CORTISOL IN SALIVA

The rate of appearance of cortisol in saliva and serum after an intravenous injection of cortisol sodium succinate was assessed in five normal adults. The apparent cortisol concentration in the saliva and serum showed an identical time course over the study period (20 min) (Fig. 1). Thus the rate of appearance of cortisol in the saliva following an increase in the blood level is very rapid, certainly much less than 5 min.

SALIVA CORTISOL V SERUM TOTAL CORTISOL

Figure 2 shows the correlation observed between salivary cortisol and serum total cortisol concentration. For normal men the correlation displays a marked change in slope at approximately 500 nmol/l of cortisol in serum. If the two sections of this plot are tested separately they each display a high correlation between the salivary cortisol concentration and the serum total cortisol concentration. The correlation for normal women appears to be the same as that for men. However, pregnant women (3rd trimester) and women using oral contraceptives form a separate group with markedly elevated serum cortisol levels in conjunction with normal salivary concentration.

Many of the data displayed in Fig. 2 for normal men were derived from the ACTH infusion studies. This technique (dexamethasone suppression followed by infusion of ACTH) proved to be a most effective way of obtaining time-matched serum and saliva samples with cortisol concentrations evenly distributed throughout the physiological range.

Figure 3 demonstrates that salivary cortisol concentration is a reliable indicator of serum unbound-cortisol concentration regardless of sex, oral contraceptive usage, or pregnancy.
The circadian variation in salivary cortisol concentration was examined in male and female adults (whose normal waking time was 0600–0730) who each collected a saliva sample every hour throughout the day. There was no significant difference between the male and female values and the data have been displayed in Fig. 4 as the mean ± 1 SD for the entire group. Salivary cortisol concentration showed the expected circadian rhythm with elevated levels shortly after rising and a nadir in the evening.

It should be noted that the normally low salivary cortisol levels in the late afternoon and evening may be grossly elevated by excitement or its anticipation. Thus we have omitted, from the estimation of mean ± SD, data from two of the normal males who had peak salivary cortisol concentrations of 19 and 45 nmol/l at 1900 and 1800, respectively, in anticipation of amateur sporting activities.

The efficacy of salivary versus serum measurements of cortisol circadian rhythm was assessed in time-matched samples of saliva and serum collected from the above group of normal subjects on a different day from the above study (so that the stress of venepuncture would not bias the subsequent data on that day). The two sampling times (0900 and 1700) were chosen as being typical of the collection times for genuine patients undergoing studies of adrenal function. Both men and women showed the expected circadian rhythm (Table 2) and there was no significant difference in the ratio (0900 value/1700 value) derived from serum or saliva. This ratio of approximately 2 is small compared with the 8-fold variation in salivary cortisol concentration between 0700 and 2000.

**Table 2 Circadian variation in cortisol in normal men and women**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Cortisol (nmol/l)</th>
<th>Men (n=5)</th>
<th>Women (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saliva Serum</td>
<td>Saliva Serum</td>
<td></td>
</tr>
<tr>
<td>0900</td>
<td>9.6±2.6</td>
<td>396±85</td>
<td>10.8±2.7</td>
</tr>
<tr>
<td>1700</td>
<td>5.7±1.3</td>
<td>259±87</td>
<td>5.8±2.8</td>
</tr>
<tr>
<td>Ratio (0900/1700)*</td>
<td>1.75±0.64</td>
<td>1.66±0.60</td>
<td>2.14±0.71</td>
</tr>
<tr>
<td>0700</td>
<td>25.5±16.0</td>
<td>21.0±7.1</td>
<td>21.0±7.1</td>
</tr>
<tr>
<td>2000</td>
<td>3.4±1.3</td>
<td>2.68±0.66</td>
<td>2.68±0.66</td>
</tr>
<tr>
<td>Ratio (0700/2000)*</td>
<td>7.1±6.1</td>
<td>8.4±3.7</td>
<td>8.4±3.7</td>
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</tbody>
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*The mean and SD for the ratios were calculated from the individual data pairs.
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Table 3  Dexamethasone suppression of cortisol

<table>
<thead>
<tr>
<th>Cortisol (nmol/l)</th>
<th>Control</th>
<th>Dexamethasone suppressed</th>
<th>Mean-ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>control</td>
</tr>
<tr>
<td>Normal men (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>378±83</td>
<td>24.2±6.4</td>
<td>17.5±7.0</td>
</tr>
<tr>
<td>Saliva</td>
<td>9.4±2.8</td>
<td>0.30±0.08</td>
<td>18.6±5.1</td>
</tr>
<tr>
<td>Normal women (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>404±40</td>
<td>72±66</td>
<td>8.3±4.0</td>
</tr>
<tr>
<td>Saliva</td>
<td>11.8±4</td>
<td>0.90±0.66</td>
<td>15.4±7.4</td>
</tr>
</tbody>
</table>

*1 mg dexamethasone (oral) at 2200. Serum and saliva collected at 0900 the following day. †average of the individual’s ratios.

22.9 nmol/l, respectively) and serum total cortisol (702 ± 93 and 779 ± 136 nmol/l, respectively).

In two patients with adrenal insufficiency salivary cortisol did not exceed 2.7 nmol/l and serum cortisol did not exceed 23 nmol/l in the 2 hours following ACTH injection (250 µg im).

The suppression of cortisol concentration in both serum and saliva after an overnight dexamethasone test was assessed in normal men and women (Table 3) and compared with the cortisol concentration on a normal morning (controls). The ratio (control:dexamethasone suppressed) for saliva cortisol concentration in men and women was not significantly different from the serum ratio.

Discussion

The biological activity of cortisol in blood is believed to be a function of the small fraction (1-15%) of cortisol that is not bound to the serum proteins, corticosteroid-binding globulin (CBG), and albumin. Unfortunately, the methods that have been developed to measure the serum unbound-cortisol level, such as equilibrium dialysis, pressure or centrifugal ultrafiltration, gel filtration and free-cortisol index are either very time-consuming, require large amounts of sample, or are of questionable accuracy.

Our results show that the salivary cortisol concentration accurately reflects the serum unbound-cortisol concentration throughout the physiological concentration range.

The line of best fit describing the relationship between salivary and serum unbound cortisol (Fig. 3) has a slope of 0.65. Thus the salivary level is about one-third lower than the serum unbound level. This value is in close agreement with the data of Umeda et al.,11 who found a slope of 0.68 though their data are largely based on supraphysiological cortisol levels induced by intravenous cortisol injection. The difference between the salivary and the serum unbound-cortisol levels is probably due to
The interpretation of serum cortisol levels is complicated by the presence of CBG, which binds cortisol with high affinity. CBG has a relatively low concentration, and its cortisol binding capacity is readily saturated at physiological cortisol concentrations. The CBG concentration is affected by various drugs (markedly increased by oral contraceptives) and physiological conditions (eg, markedly increased by pregnancy). Thus the relationship of salivary to serum cortisol concentrations in our female subjects was not significantly different from that of the males in the low cortisol region (<400 nmol/l of serum). However, in the high serum cortisol region (>600 nmol/l) the pregnant women (third trimester) and the women who had been on oral contraceptives for more than one year were well removed from the men. They often had markedly elevated serum cortisol levels (700–1200 nmol/l) with concurrent normal levels of salivary cortisol and serum unbound cortisol (10–30 nmol/l) (Fig. 2). These observations are consistent with the well-known effect of pregnancy and of oestrogen therapy in increasing the concentration of CBG.

Clearly any test of adrenal function that involves a change in cortisol level from below to above (or vice versa) the saturation level for CBG will involve a larger apparent response in salivary or serum unbound cortisol than in serum cortisol. This effect was very evident in the ACTH stimulation tests where the proportional increase in salivary and serum unbound cortisol levels was 5 to 10 times greater than the increase in serum cortisol (Fig. 6) because of the saturation of CBG (at 400–500 nmol/l in normal men and women) and the consequent rapid increase in the unbound cortisol concentration.

In contrast, dexamethasone suppression tests and tests of circadian variation (0900–1700) only involve a variation of cortisol concentration which is within the binding capacity of CBG, and accordingly the salivary cortisol and serum unbound cortisol response is comparable to the serum cortisol response.

Serum sampling times for hospital outpatients are generally restricted to office hours, and accordingly tests of circadian variation are often based on samples taken at 0900 and 1700 when the ratio is only approximately 2 in normals and may be readily masked by the stress of the clinic visit and venepuncture. The stress-free, easy sample collection procedure makes it possible for patients to collect their own samples at times closer to the peak and nadir of their circadian rhythm (the ratio for saliva samples collected at 0700 and 2000 in normals is approximately 8), which should provide clinicians with less ambiguous data on circadian variation.

Our studies of circadian variation in pregnancy showed that the markedly elevated cortisol levels typical of late pregnancy were not due entirely to higher concentrations of CBG. The salivary cortisol levels appeared to be elevated throughout the day, and this elevation was statistically significant in the afternoon, a finding consistent with other recent studies.17 18

For our studies of the rate of appearance of cortisol in saliva we were unable to obtain pure cortisol suitable for human use, and consequently we used cortisol sodium succinate (BP). The succinate moiety of the conjugate is cleaved by the liver to yield cortisol, which is the primary steroid detected by our cortisol RIA; however, cortisol sodium succinate does show a 20% cross reaction in the assay. Accordingly, we have not used data from this experiment in our correlations of salivary and serum cortisol levels. However, the data from this experiment are quite adequate for determining the rates of appearance of cortisol in saliva, which is very fast, being less than 5 min.

The use of measurements of salivary cortisol concentration as a measure of adrenal cortical function in preference to the more usually measured serum cortisol has a number of advantages. Firstly, the salivary cortisol concentration reflects the biologically active serum unbound cortisol level and is thus unaffected by elevations in CBG, which confuse the interpretation of serum cortisol levels. Also, saliva is easy to collect and the collection procedure is non-invasive and stress free, making it ideal for use in pregnant women or in children. Similarly, the ease of collection facilitates studies requiring the collection of multiple samples which may reduce the confusing effects of episodic secretion upon the interpretation of steroid hormone levels.

We wish to thank particularly the many volunteers who collected samples for this project. Our thanks are also due to Ms J Robinson for arranging collection of some of the samples, the Wrigley Company for supplying unflavoured chewing gum, Ms B Rice and Mr R Lawson-Smith for skilful technical assistance, and Mr P Compton for statistical advice.

References

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