Advantage of salivary cortisol measurements in the diagnosis of glucocorticoid related disorders

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Abstract

Objective: Salivary cortisol in the assessment of glucocorticoid related disorders.

Design-methods: Serum and salivary cortisol were measured in 189 patients (22 Cushing’s syndrome, 67 pseudo-Cushing, 11 Addison’s disease, 89 controls) at 8:00 and 24:00 h.

Results: Serum and salivary cortisol correlated in the whole study population (r=0.62, p=0.000). Morning serum and saliva cortisol in Addison’s disease were lower than in controls (6.74±1.69 vs 22.58±1.78 µg/dL, and 0.15±0.25 vs 0.67±0.12 µg/dL) (p<0.001). Morning serum cortisol was similar in controls and patients with Cushing’s syndrome or pseudo-Cushing (22.58±1.78 vs 13.96±6.02 vs 16.13±1.69 µg/dL). Morning serum and salivary cortisol at 8:00 had the same sensitivity to distinguish patients with Addison’s disease from healthy controls. 24:00am serum cortisol in controls (2.61±0.20 µg/dL) was lower than in the pseudo-Cushing group (6.53±0.77 µg/dL, p<0.001) and in Cushing’s syndrome (10.90±2.36 µg/dL, p=0.003). 24:00am salivary cortisol in controls (0.0025±0.001 µg/dL) was lower than in patients with Cushing’s syndrome (0.58±0.11 µg/dL, p<0.001) and those higher than in patient with pseudo-Cushing (0.10±0.06 µg/dL, p=0.001).

Both salivary cortisol and serum cortisol presented high specificity (82% and 100%) to detect Cushing’s syndrome but salivary cortisol higher sensitivity (saliva 88% and serum 50%).

Conclusion: Morning salivary cortisol is as good as serum as screening test for patients with Addison’s disease and nighttime salivary cortisol is more adequate than serum in the screening of Cushing’s syndrome.

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Keywords: Cortisol; Saliva; Serum; Addison’s disease; Cushing’s syndrome; Pseudo-Cushing; Glucocorticoids; Screening; Sensitivity; Specificity

Introduction

The diagnosis of disorders related to alteration in glucocorticoids plasma is sometimes difficult because symptoms are not specific and unfortunately, all currently available biochemical tests have limitations and there is controversy about which test is the most appropriate for the different situations [7].

Over 90% of the cortisol in the circulation is bound to proteins (85% to transcortine and 10% to albumin) and the biological activity depends exclusively on the free fraction [4]. The usual practice is to measure the total concentration (free and bound to proteins) which is therefore not representative of the active form. Cortisol measured in 24 h-urine samples is a better approximation as only free cortisol is excreted by urine [5]. However, urine measurements may be inaccurate due to inadequate collection [6].

Another limitation for the diagnosis of glucocorticoid related disorders is that plasma cortisol levels present circadian rhythm with the lowest concentration at midnight, rising to a peak between 6:00 and 8:00 h, and falling throughout the rest of the day. Thus, dynamic laboratory tests are necessary to confirm the diagnosis [7].

One of the most sensitive screening strategies to discard hypercortisolism is serum cortisol at 24:00 h [8], albeit other studies recommend the quantitation of 24 h urine free cortisol [9]. This procedure is especially recommended for the differential
diagnosis between Cushing’s syndrome and pseudo-Cushing. The dynamic tests low-dose dexamethasone suppression or the dexamethasone-CRH test, despite their limitations, are employed for confirmation of endogenous Cushing’s syndrome [10].

Salivary cortisol measurements present a number of advantages to overcome these technical problems as cortisol in saliva is present only in the biologically active form [11,12]. Also, bedtime serum cortisol levels require the admission of the patient in the hospital to obtain a sample of blood. These results may be misinterpreted because venipuncture generates stress that raises cortisol plasma concentration. On the contrary, the determination of salivary levels of cortisol allows patients to obtain the sample at home by a non-invasive procedure. In spite of salivary cortisol presenting more advantages than serum cortisol, it is not used in clinical practice as much as serum cortisol, due to the relatively lack of publications [13] and standardized cut-off points for the initial screening.

Thus, the aims of this work were to study 1) whether cortisol levels in serum and in saliva correlate in healthy individuals and patients with different glucocorticoid related pathologies, 2) salivary cortisol in the initial screening of glucocorticoid related disorders with a proposal of reference values in saliva and 3) the sensitivity, specificity and cut-off values of salivary and serum cortisol for predicting Addison’s disease and Cushing’s syndrome.

**Subjects and methods**

**Study population**

The study population consisted of 11 patients (5 women, 51±12 years), attending the Department of Endocrinology diagnosed of Addison’s disease based on methods of reference. All patients were on replacement therapy with hydrocortisone. None of the patients was treated with other synthetic glucocorticoids. Twenty-two subjects were diagnosed with Cushing’s syndrome (13 women and 9 men; 53±16 years) based on methods of reference.

The control group consisted of 89 healthy individuals (55 women, 39±16 years). In addition, patients with diverse pathologies that alter cortisol levels were included in the study (n=67, 31 women, 55±17 years, pseudo-Cushing group). In this group, in spite that the term pseudo-Cushing is controversial [2,3], we grouped obese patients [5], patients with depression [3] and patients who underwent cardiac bypass surgery (55).

 Patients with kidney or liver disease were excluded from the study.

All procedures were performed in accordance with the Declaration of Helsinki. All study participants signed the informed consent and the local ethics committee approved the study.

**Sample acquisition**

Patients did not take their medication for at least 24 h (the biological half-life of hydrocortisone is 8–12 h and the plasmatic half-life 120 min) and serum and salivary cortisol levels at 8:00 and 24:00 h were measured in patients with Cushing’s syndrome, controls and pseudo-Cushing patients. In patients with Addison’s disease only morning measurements were employed.

Serum samples were collected by venous puncture from all study participants. Patients were admitted in the hospital and samples at midnight were obtained while sleeping. Samples were immediately centrifuged (3500 rpm, 7 min) and serum stored at −20 °C until processing.

Salivary cortisol was collected in special tubes (LCL Laboratoire) consisting of a small tube, which contains a cotton wool, contained into a bigger tube. Patients cleaned their mouth, did not eat, drink or smoke for 1 h and rinsed their mouth with cold water just before obtaining the sample. The cotton wool was chewed for 2–3 min, placed again into the small tube and kept at 4 °C until carried to the laboratory [14,15].

Salivary samples were centrifuged (3500 rpm, 10 min) to make saliva fall from the cotton to the biggest tube. After centrifugation, saliva was stored at −20 °C until processing.

Serum and salivary cortisol were obtained in consecutive days.

**Cortisol measurement in serum and saliva**

Serum cortisol was measured in the Immulite 2000 (Dipesa, Los Angeles, USA) by a competitive chemoluminiscence assay which measures total cortisol in serum because the antibodies of this technique are capable of binding free cortisol and that bound to transcortin or albumin. The calibration range of the assay was 1–50 µg/dL, with an analytical sensitivity of 0.20 µg/dL and a coefficient of variation inter-assay and intra-assay of 6.8% and 5.2%, respectively.

Salivary cortisol was measured by ELISA (Diagnostic Systems Laboratories) as indicated by the manufacturer. The calibration range of the assay was 0.1–10.0 µg/dL, with an analytical sensitivity of 0.001 µg/dL and coefficient of variation inter-assay and intra-assay of 2.8% and 2.8%, respectively.

**Data analysis**

The statistical analysis was performed using the SPSS 13.0. Normal distribution of variables was assessed by the Shapiro–Wilkst test. Correlations were studied by the Spearman test. The student T test was employed to compare differences between groups. Receiver operating characteristic (ROC) curves allowed the determination of the overall performance of serum and salivary cortisol levels for predicting Cushing’s syndrome or Addison’s disease. The Youden Index was calculated for the selection of the optimal cutoff points. All data are expressed as mean±standard deviation. All p-values were two tailed and significance was established at p<0.05.

**Results**

**Cortisol levels in serum and in saliva**

Cortisol concentrations in serum significantly correlated with cortisol concentrations in saliva in the whole studied population (r=0.62, p=0.000) (Fig. 1). The correlation between serum and
Salivary cortisol is also observed when evaluating separately healthy people ($r = 0.67$, $p = 0.050$), patients with Addison’s disease ($r = 0.73$, $p = 0.011$), patients with pseudo-Cushing ($r = 0.55$, $p = 0.001$) and patients with Cushing’s syndrome ($r = 0.61$, $p = 0.003$).

Morning serum cortisol levels in patients with Addison’s disease were significantly ($p < 0.001$) lower than in controls (6.74 ± 1.69 vs 22.58 ± 1.78 µg/dL, respectively). Also, salivary cortisol levels at 8:00 h were significantly ($p = 0.002$) lower in patients with Addison’s disease (0.15 ± 0.25 µg/dL) than in controls (0.67 ± 0.12 µg/dL) (Table 1).

Morning serum cortisol levels do not differ between controls and patients with Cushing’s syndrome or pseudo-Cushing (22.58 ± 1.78 vs 13.96 ± 6.02 vs 16.13 ± 1.69 µg/dL; respectively) (Table 1). Also, there are no statistically significant differences in morning salivary cortisol levels between controls, patients with pseudo-Cushing and patients with Cushing’s syndrome (0.67 ± 0.12 vs 0.95 ± 0.13 vs 1.05 ± 0.50 µg/dL; respectively).

24:00 am serum cortisol levels in controls (2.61 ± 0.20 µg/dL) were lower than in the pseudo-Cushing group (6.53 ± 0.77 µg/dL, $p < 0.001$) and in patients with Cushing’s syndrome (10.90 ± 2.36 µg/dL, $p = 0.003$). However, serum cortisol concentrations in patients with Cushing and pseudo-Cushing did not differ (10.90 ± 2.36 vs 6.53 ± 0.77 µg/dL, $p = 0.095$).

24:00 am salivary cortisol concentrations in controls (0.0025 ± 0.001 µg/dL) were lower than in patients with Cushing’s syndrome (0.58 ± 0.11 µg/dL, $p < 0.001$). But, in contrast to serum cortisol, there were no statistical differences in salivary cortisol between controls and patients with pseudo-Cushing (0.0025 ± 0.001 vs 0.10 ± 0.06 µg/dL; respectively, $p = 0.137$). Importantly, 24:00 am salivary cortisol levels in patients with Cushing’s syndrome were higher than in patient with pseudo-Cushing (0.58 ± 0.11 vs 0.10 ± 0.06 µg/dL; respectively, $p = 0.001$).

### Overall analytical performance of salivary cortisol levels

To study the analytical performance of cortisol measurements in the diagnosis of Addison’s disease and Cushing’s syndrome, the cut-off values and the corresponding analytical sensitivity and specificity for the two parameters were calculated according to the ROC curves.

The area under the ROC curves for morning salivary and serum cortisol levels for the prediction of Addison’s disease were lower than 0.5 (0.27 and 0.16, respectively). Serum and salivary cortisol at 8:00 h had the same sensitivity to distinguish patients with Addison’s disease from healthy controls (Table 2). However, morning serum cortisol presented higher specificity to detect patients with Addison’s disease.

The area under the ROC curves for the predictions of Cushing’s syndrome were lower than 0.5 for morning salivary and serum cortisol (0.45 and 0.41, respectively). However, the area under the ROC curves for 24:00 am salivary and serum cortisol for the prediction of Cushing’s syndrome were higher than 0.5 (0.87 and 0.62, respectively) (Fig. 2).

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**Table 1** Morning and midnight serum and salivary cortisol levels in the control group, patients with Addison’s disease, patients with pseudo-Cushing and patients with Cushing’s syndrome.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Addison’s disease</th>
<th>Pseudo-Cushing</th>
<th>Cushing’s syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n=89$</td>
<td>$n=11$</td>
<td>$n=67$</td>
<td>$n=22$</td>
</tr>
<tr>
<td>Serum cortisol (8:00 am) (µg/dL)</td>
<td>22.58±1.78</td>
<td>6.74±1.69*</td>
<td>16.13±1.69</td>
<td>13.96±6.02</td>
</tr>
<tr>
<td>Salivary cortisol (8:00 am) (µg/dL)</td>
<td>0.67±0.12</td>
<td>0.15±0.25*</td>
<td>0.95±0.13</td>
<td>1.05±0.50</td>
</tr>
<tr>
<td>Serum cortisol (24:00 am) (µg/dL)</td>
<td>2.61±0.20</td>
<td>–</td>
<td>6.53±0.77*</td>
<td>10.90±2.36*</td>
</tr>
<tr>
<td>Salivary cortisol (24:00 am) (µg/dL)</td>
<td>0.0025±0.001</td>
<td>–</td>
<td>0.10±0.06</td>
<td>0.58±0.11*†</td>
</tr>
</tbody>
</table>

* $p < 0.01$ vs control group.
† $p < 0.001$ vs pseudo-Cushing group.

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**Table 2** Overall performance of morning serum and salivary cortisol levels for predicting Addison’s disease.

<table>
<thead>
<tr>
<th>Time</th>
<th>Cut off (µg/dL)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 8:00 h</td>
<td>11.9</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td>Salivary 8:00 h</td>
<td>1.53</td>
<td>33</td>
<td>20</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Cortisol levels measured in samples of serum correlate ($r=0.618$, $p=0.000$) with those found in saliva obtained at the same time point from 89 healthy people, from 11 patients with Addison’s disease, from 67 patients with pseudo-Cushing and from 22 patients with Cushing’s syndrome.

**Fig. 2.** ROC analysis of 24:00 am serum and salivary cortisol levels in the diagnosis of Cushing’s syndrome.
serum cortisol at 8:00 h presented similar specificity but salivary measurements showed higher sensitivity (40% vs 20%) (Table 3). However, the more relevant difference is found at 24:00 h when both salivary cortisol and serum cortisol present high specificity (82% and 100%, respectively) but salivary cortisol much higher sensitivity (saliva 88% and serum 50%).

Discussion

Serum and salivary cortisol levels in Addison's disease and Cushing's syndrome

Measurement of serum cortisol is the common practice for the initial screening of Addison’s disease and Cushing’s syndrome. Interestingly, in the present study, serum and salivary cortisol levels significantly correlated in 189 individuals, both in healthy individuals and in patients with different glucocorticoid related disorders. This finding is in agreement with previous reports and shows, as other groups have also demonstrated, that the association of serum and salivary cortisol appears to be independent of the status of health of the patient [1,16,17].

As expected, serum cortisol levels were different in patients with Addison’s disease or Cushing’s syndrome compared with controls and these differences are reproduced in saliva. Both serum and saliva cortisol levels are lower in patients with Addison’s disease, compared with healthy subjects suggesting that saliva measurements are appropriate in handling this disease. Patients with Cushing’s syndrome or pseudo-Cushing present higher serum levels of cortisol at midnight than controls without differences between patients with Cushing’s syndrome or pseudo-Cushing. One important and relevant finding of the present study for the clinical practice is that salivary cortisol levels at 24:00 h allows distinguishing patients with Cushing’s syndrome from patients with pseudo-Cushing. Based on our data, healthy controls present suppressed values of cortisol at midnight. People with pathologies that alter cortisol levels different from Cushing’s syndrome have detectable levels of salivary cortisol at 24:00 h, but those levels are similar to levels of healthy controls and significantly lower than in patients with Cushing’s syndrome.

Analytical performance of cortisol salivary measurements in the diagnosis of Addison’s disease and Cushing’s syndrome

We next studied the analytical characteristics of serum and salivary cortisol levels in the diagnosis of patients with Addison’s disease or Cushing’s syndrome.

<table>
<thead>
<tr>
<th>Time</th>
<th>Cut off (µg/dL)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 8:00 h</td>
<td>26</td>
<td>20</td>
<td>86</td>
</tr>
<tr>
<td>Salivary 8:00 h</td>
<td>1.98</td>
<td>40</td>
<td>89</td>
</tr>
<tr>
<td>Serum 24:00 h</td>
<td>10.54</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Salivary 24:00 h</td>
<td>0.08</td>
<td>88</td>
<td>82</td>
</tr>
</tbody>
</table>

Table 3
Overall performance of morning and midnight serum and salivary cortisol levels for predicting Cushing’s syndrome

Serum cortisol at 8:00 h is currently used in the clinical practice for the diagnosis of Addison’s disease. According to our data this measurement presents the same sensitivity than salivary cortisol, suggesting that saliva is an appropriate alternative to serum samples. Results from individual patients should be interpreted with care because for both samples the specificity of the test is relatively low, however acceptable for a screening test.

According to our data, morning cortisol measurements do not appear to be a proper analysis in the evaluation of Cushing’s syndrome due to the relatively low sensitivity, in spite of saliva presenting higher sensitivity that serum. On the contrary, bedtime measurements showed that salivary cortisol levels have higher sensitivity than serum levels, while the specificity is similar [18]. Thus, salivary cortisol levels could be used as a first line screening test for Cushing syndrome in the general population because of the high sensitivity with also a very good specificity that reduces the number of false positives. These findings are in agreement with other studies that have found slightly higher sensitivities, also employing healthy people as controls [19]. There are other studies that employ healthy people and obese patients as control group [20–22]. Other studies have also concluded that salivary levels at midnight are an adequate screening test, but they argue a lack of validation of diagnostic criteria [23]. Thus, we present reference values, obtained from patients with Cushing’s disease, healthy people and patients with pseudo-Cushing, in order to obtain representative values of the general population. Therefore, 0.08 µg/dL in saliva appears to be an appropriate limit to distinguish patients with Cushing’s syndrome and perform subsequent confirmation. Other groups have published higher levels of cortisol [14], but the collection method affects the results, thus values are not comparable. Moreover, other groups have reported reference values for salivary cortisol employing RIA [26]. The reference values shown here are obtained by ELISA, which is an easier and safer technique compared to RIA which employs radioactivity.

Advantages of salivary measurements

Thus, as salivary measurements present similar or better analytical characteristics in the detection of patients with Cushing’s syndrome, our data also support that it is possible to replace the serum determination or even dynamic function tests [24] with salivary cortisol measurements as a screening test, because they present several advantages. First, in addition to eliminating venipuncture, saliva can be easily obtained at home, avoiding admission in the hospital. Secondly, rapid changes in cortisol levels happen in the hour or so after waking. Thus, reducing the amount of time between waking up and sample collection may have an impact on the results. Salivary cortisol measurements could minimize this bias improving test performance. Furthermore, most currently screening tests for Cushing’s syndrome have the disadvantage of a high false positive rate. In our study we have shown that in patients with Cushing’s syndrome, bedtime salivary cortisol presents higher sensitivity than serum cortisol, which could be due to the fact that salivary levels of cortisol reflect the biologically active free form. Moreover, salivary cortisol measurements allowed
distinguishing between patients with Cushing’s syndrome and pseudo-Cushing. Thus, overall the rate of false positive results is reduced avoiding unnecessary tests [25].

One limitation of this study is that we determined salivary cortisol by ELISA, which could differ from results obtained by other techniques, although there are experiments that support determinations by RIA and EIA correlated [26].

In summary, in this study we have shown that morning salivary cortisol is as good as serum as an initial screening test of the diagnosis of patients with Addison’s disease. In addition, bedtime salivary cortisol levels, due to their accuracy and convenience are a valuable alternative to help in the diagnosis of patients with Cushing’s syndrome.

References


