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Use of salivary cortisol and cortisone in the high and low dose synacthen test

Short title: salivary cortisone following synacthen

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Summary: Salivary cortisone closely correlates with serum cortisol in the unstimulated state and after 50 minutes following synacthen stimulation. We recommend a 60min sample be used for diagnostic accuracy.

Keywords: ACTH stimulation test, Synacthen, cosyntropin, saliva, cortisol, cortisone, adrenal insufficiency, Addisons

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Abstract

Context: Salivary cortisone reflects serum cortisol levels, is more sensitive than salivary cortisol at lower values of serum cortisol and is non-invasive.

Objective: To investigate the relationship between serum cortisol and salivary cortisol and cortisone following low and high-dose synacthen.

Design and Setting: Prospective pharmacodynamic studies in clinical research facilities.

Participants and Intervention: 35 dexamethasone-suppressed, healthy adult males underwent an intravenous synacthen test: N=23 low-dose (1mcg), N=12 high-dose (250mcg). Paired serum and salivary samples were taken at 15 sampling points over 120 minutes.

Main outcome measure: Serum cortisol and salivary cortisol and cortisone were analyzed for correlations and by a mixed effects model.

Results: At baseline the correlation between serum cortisol and salivary cortisol was weak with many samples undetectable (r=0.45, NS), but there was a strong correlation with salivary cortisone (r=0.94, p<0.001). Up to 50 minutes following synacthen the correlation coefficient between serum cortisol and salivary cortisol and cortisone was <0.8, but both had a stronger correlation at 60 minutes (salivary cortisol r=0.89, p<0.001, salivary cortisone r=0.85, p<0.001). The relationship was examined excluding samples in the dynamic phase (baseline to 60 minutes). Salivary cortisol and cortisone showed a close relationship to serum cortisol. Salivary cortisone showing the stronger correlation: Salivary cortisol r=0.82, p<0.001, salivary cortisone r=0.96, p<0.001.

Conclusion: Following synacthen, both salivary cortisol and cortisone reflect serum cortisol levels but there is a lag in their rise up to 60mins. The results support further research for possible future use of a 60min salivary cortisone measurement during the synacthen test.

Introduction

Measurement of salivary cortisol is becoming routine in some clinics for the assessment of adrenal function. Late-night salivary cortisol testing is now recommended as a first-line diagnostic test in Cushing's syndrome [1], and there are reports of its use in the diagnosis of adrenal insufficiency [2-10]. Advocates of salivary measurements favor its convenience and potential cost savings over serum sampling. It is non-invasive, painless, has no requirement for clinic attendance, reflects serum free

cortisol and there is less risk of false positive results, generated by the cortisol stress response to venipuncture, when testing for Cushing's disease. Recent evidence suggests that salivary cortisone is a better reflection of serum total and free cortisol than salivary cortisol [2,11-13]. Serum free cortisol and salivary cortisol are rapidly oxidized to inactive cortisone, by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2), which is responsible for the large difference in the proportion of salivary cortisol:cortisone (1:6) compared with that in serum (4:1) [11]. Salivary cortisone reflects serum cortisol levels both under physiological conditions [2] and after administration of hydrocortisone, with 94% of the variability in salivary cortisone attributable to changes in serum cortisol [11,12]. Salivary cortisone is more sensitive at low serum cortisol levels than salivary cortisol making it better suited for the detection of adrenal insufficiency [2,5,8,12].

Stimulation of the adrenal cortex with synthetic (1-24) ACTH (synacthen) is the standard diagnostic test for adrenal insufficiency [9,14,15]. The test involves administration of 250 mcg of synacthen, a supraphysiological dose. The low dose synacthen test (most commonly 1 mcg) is used by some clinicians, as it is thought to more closely mimic a physiological stress stimulus to the adrenal cortex [14]. Results of meta-analyses do not show significant superiority of one test over another and both doses are used in clinical practice [16-20].

The measurement of salivary cortisol has been used after stimulation, with different doses of synacthen, in both healthy volunteers and patients [3-6,8,10]. The results support the use of salivary sampling after synacthen, particularly in groups with low serum steroid binding-protein levels and women on estrogens [10,12,21]. There is limited data on the use of salivary cortisone following synacthen stimulation [5,8,12]. We report the relationship of serum cortisol, salivary cortisol and salivary cortisone following administration of low and high dose synacthen and demonstrate the importance of sample timing.

Subjects and Methods

Three prospective pharmacodynamic studies were conducted at the Clinical Research Facilities of Sheffield Children's NHS Foundation Trust and Sheffield Teaching Hospitals NHS Trust. The studies recruited 35 healthy adult males, aged 19-46 years (median 22, IQR 21.5-23.5), BMIs (19.1-29.4 kg/m^2 (median 23.2, IQR 21.8-24.4). Volunteers were excluded if they smoked, had been diagnosed with an endocrinopathy, intra-cranial or adrenal pathology, asthma, allergic rhinitis, anemia, peptic ulcer disease, gastrointestinal bleed or dyspepsia, experienced a severe allergic reaction or any hypersensitivity to synacthen, were on any regular or prescribed medication, received any formulation of corticosteroid in the previous three months or had ever had a course of oral corticosteroids lasting more than one month. Study 1 was approved by Leeds (West) Research Ethics Committee and studies 2 and 3 by London-Hampstead Research Ethics Committee. Written informed consent was given by all participants.

Procedures: The studies were conducted in a similar fashion. All visits commenced between 08.30 and 09.30. Following basic demographic and auxological data collection volunteers had an intravenous cannula sited and then rested for 30 minutes. They were asked to remain supine for the duration of the testing. Following baseline samples volunteers received intravenous synacthen (250 mcg/ml vials, Mallinckrodt Specialty Pharmaceuticals Ireland Ltd, Dublin, Ireland). In the low-dose studies volunteers (N=23) received 1 mcg of intravenous synacthen by diluting 0.5 ml of 250 mcg/ml solution in 500 ml of 0.9% saline, mixing thoroughly and administering 4 ml of the resultant solution (low-dose test). In the high-dose study (N=12) volunteers received 250 mcg of synacthen (high-dose test). Paired blood and saliva samples were taken at the following times (administration of synacthen at 0 minutes): -1, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, and 120 minutes, with replacement of the 15 minute sample with a 2 minute sample in the high-dose study. All blood samples were taken from the indwelling cannula. Saliva sampling was performed using the passive drool technique, which involves volunteers spitting or drooling down a straw into a salicap tube (IBL Hamburg, Germany) for collection. Ten minutes before the first samples were taken volunteers were asked to rinse their mouth thoroughly with water and did not eat or drink anything (other than water) until conclusion of

the study visit. Saliva sampling was done at the same time point as serum sampling with subject initiating the drool at the same time as the syringe was connected to the cannula to withdraw the discard before taking the serum sample. To provide a uniform baseline all volunteers were dexamethasone suppressed prior to each visit with 1 mg on retiring the night before and a further 1 mg after breakfast on the morning of the test. Successful dexamethasone suppression was established by undetectable ACTH and cortisol at baseline (-1 minute sample).

Assays: Serum cortisol samples were analyzed using the Abbott Architect chemiluminescent microparticle immunoassay (Abbott Diagnostics Ltd, Berkshire, UK). The minimum detectable dose of cortisol for the assay is <0.8 mcg/dL (22 nmol/L) with a functional sensitivity of <1 mcg/dL (27 nmol/L). It has an assay precision of 4.0% total CV for serum samples <3 to 35 mcg/dL (82 to 965 nmol/L). The quoted cross-reactivity with cortisone is 2.7%. Salivary cortisol and cortisone analysis was performed by a modified liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay using a Waters Xevo TQ-MS mass spectrometer and a Waters Acquity LC system with an electrospray source operated in positive-ionization mode [22]. Lower limits of quantitation were 0.03 mcg/dL (0.8 nmol/L) for both salivary cortisol and salivary cortisone and intra-assay CVs of 9.3% and 7.9%, and inter-assay CVs of 9.7% and 10.3% at <0.1-1.9 mcg/dL (1.8-52.2 nmol/L) of salivary cortisol and 0.1-3.5 mcg/dL (3.6-96 nmol/L) of cortisone, respectively.

Statistical analysis:

T-tests were used to compare the salivary cortisol and salivary cortisone Cmax (peak serum concentration achieved) and Tmax (time at which peak serum concentration achieved) following low-dose coyntropin and $p<0.05$ considered significant. A linear mixed-effects model relating the (logarithm of) serum cortisol to each of (logarithm of) salivary cortisol and salivary cortisone was computed. The random effect was taken to be the subject. Pearson correlations were computed between (logarithm of) serum cortisol and the logarithms of each of salivary cortisone and salivary cortisol. Samples below the level of detection for serum and salivary assays were excluded from model analysis: 25 serum cortisol samples <0.8 mcg/dL (22 nmol/L), 62 salivary cortisol <0.1 mcg/dL

(0.8 nmol/L) and one salivary cortisone <0.1 mcg/dL (0.8 nmol/L). All computation was carried out using the Matlab (R2016b) numerical computing environment (Mathworks, Natick, MA).

Results

Response to synacthen (Figure 1 and Table 1): Salivary cortisol and cortisone demonstrated similar time concentration profiles to serum cortisol following stimulation with high-dose (250 mcg) and low-dose (1 mcg) synacthen. The rise in corticosteroids showed a different pattern after high-dose compared with low-dose synacthen with the median serum cortisol Cmax higher (22 and 14 mcg/dL respectively (615 and 386 nmol/L)), and Tmax later (120 and 30 mins respectively). There was a difference in Tmax for salivary cortisol and cortisone following low-dose synacthen 30 vs 40 mins respectively (p=0.001). The mean serum concentration of cortisol following 250 mcg synacthen at 30 minutes was 15 mcg/dL (407 nmol/L) (SD 44, range 349-477) and at 60 minutes 19 mcg/dL (511 nmol/l) (SD 49, range 433-581). Salivary cortisol was <0.8 nmolL in 16.2% of samples at low serum cortisol levels, whereas salivary cortisone was detectable in all but one sample, which was collected at 5 minutes with a corresponding salivary cortisol of <0.8 nmol/L and serum cortisol of 0.9 mcg/dL (25 nmol/L).

Relationship between serum and salivary observations (Figure 2): Linearity between variables was achieved after log transformation and both salivary cortisol and cortisone correlated with serum cortisol, however it was evident that at the early time points, following synacthen, serum cortisol was relatively higher than the salivary measurements. We therefore examined the relationship excluding the samples between baseline and 60 minutes. In this analysis both salivary cortisol and salivary cortisone showed a close relationship to serum cortisol, with salivary cortisone showing the stronger correlation: Salivary cortisol r=0.82, p<0.001 and salivary cortisone r=0.96, p<0.001. Our mixed effects model derived from these data gave the line intercept for ($\log_{10}$) salivary cortisol of 2.17 with a slope of 0.387 and 1.23 for ($\log_{10}$) salivary cortisone with a slope of 0.823. The correlation between the fixed effects prediction for the salivary cortisol model was 0.82 and for the salivary cortisone model, 0.96 (p<0.001).

We further examined the relationship between salivary cortisone and serum cortisol at each individual time point (Figure 3). From these data it can be seen that, after the baseline sample and up to 30 minutes, the value of serum cortisol falls above the regression line for the fixed effects model. At 40 minutes the serum cortisol is closer, but the majority of values lie above the regression line, and from 50 minutes to 120 minutes the serum cortisol falls on or near the regression line. When we plot the correlation between salivary cortisol and salivary cortisone with serum cortisol for the individual time points (Figure 4), we can see that at time zero there is a very poor correlation for salivary cortisol ($r=0.45$, NS) because many samples fall below the lower limit of detection, but for salivary cortisone there was a strong correlation ($r=0.94$, $p<0.001$). After synacthen the correlation between serum cortisol and salivary cortisol and salivary cortisone fell and then rose so that after 50 minutes the correlation was $r>0.8$, $p<0.001$ for both.

Discussion

Our results show that following synacthen, salivary cortisone reflects serum cortisol levels but importantly there was a lag in the rise in salivary cortisone such that up to 50 minutes salivary cortisone was lower than would be predicted from serum cortisol values. Salivary cortisone showed a stronger correlation with serum cortisol than salivary cortisol primarily because at low levels of serum cortisol, salivary cortisol was <0.8 nmol/L. The results support the use of salivary cortisone measurement during the synacthen test but suggest that rather than using a 30 minute sample, as is commonly used with serum cortisol, the measurement of salivary cortisone at 60 minutes may be a more reliable reflection of serum cortisol.

The response of serum cortisol to both 250 mcg and 1 mcg synacthen is consistent with previous reports and thus demonstrates a reliable and reproducible response [23-25]. Exposure to two doses of dexamethasone would not be expected to suppress the adrenal gland but reduces the basal cortisol level and therefore the peak serum cortisol achieved by our healthy subjects. Even with a modest early morning baseline of 4 mcg/dL (110 nmol/L) all subjects would have reached a peak cortisol of more than 18 mcg/dL (500 nmol/L) at 60 minutes and more than 16 mcg/dL (450 nmol/L) at 30 minutes (our local assay-derived diagnostic threshold).

There are concerns that, in view of the slower clearance of salivary compared with serum cortisol following IV and oral hydrocortisone administration, prior treatment with hydrocortisone may interfere with the interpretation of the synacthen test if glucocorticoid response is measured in saliva [26]. Our previous work demonstrated that the relationship between serum cortisol and salivary cortisone is the same under physiological conditions as it is after oral or iv hydrocortisone and after synacthen [11]. Prior administration of hydrocortisone may therefore impact on the test's interpretability but need not prevent the use of salivary cortisone, rather than serum cortisol, for interpretation.

Peak serum cortisol was achieved at 30 minutes following low-dose synacthen but continued to rise to the last sampling point at 120 minutes after 250 mcg synacthen. A similar pattern of response was seen with salivary cortisol and cortisone indicating a rapid transfer of free cortisol in serum to saliva. We demonstrated the same Tmax for both serum cortisol and salivary cortisol indicating that movement of free cortisol from serum to saliva is very rapid. The modal Tmax for salivary cortisone was 10 minutes later than for salivary cortisol and could relate to conversion of salivary cortisol to salivary cortisone by 11β-HSD-2.

Salivary cortisone was higher at baseline and throughout the synacthen test than salivary cortisol. Salivary cortisol was <0.8 nmol/L in 62 (16.2%) of samples and salivary cortisone only in a single sample. This may be explained by the rapid conversion of free cortisol to salivary cortisone in the salivary gland [12]. At baseline, before synacthen administration, serum cortisol and salivary cortisone exhibited a tight correlation (r=0.94), while serum cortisol and salivary cortisol were poorly correlated (r=0.45). This relates to the number of baseline salivary cortisol samples of <0.8 nmol/L excluded from analysis.

Following administration of synacthen the correlation between serum cortisol and both salivary cortisol and cortisone fell below 0.8 until the 50 minute sampling time point. Salivary cortisone is a product of both enzymatic conversion of salivary cortisol by 11β-HSD-2 and passive diffusion of free serum cortisone into saliva. Our results show that, with frequent sampling, under stimulated conditions, salivary cortisol and salivary cortisone correlate poorly with serum cortisol in the early dynamic phase of the synacthen test. This may reflect a lag between conversion of salivary cortisol to cortisone by 11β-HSD-2 but there is no evidence of enzymatic saturation. A previous study of salivary cortisol and salivary cortisone following 250 mcg synacthen demonstrated that free serum cortisone

did not rise in response to stimulation and had no influence on salivary cortisone fluctuations [12]. The challenges of obtaining accurately paired salivary and blood samples, performed at very close intervals in order to model the pharmacodynamics of the response to synacthen, may also give rise to a reduced correlation, most obvious during the early frequent sampling times.

The mixed effects model for serum cortisol and salivary cortisone applied to our data is similar to that previously published [11]. The line intercepts are the same (our data 1.23, published model 1.24) but slopes differ by a small degree (our data 0.823, published model 0.981, $p<0.001$) [11]. The difference may reflect the different assay techniques employed to quantify serum cortisol [27]. The published model used LC/MS-MS where in this study we used an immunoassay [11].

The limitations of this study include: (i) the study was carried out on healthy individuals, (ii) that dexamethasone administration may interfere with the interpretation of the test, (iii) broad-based assay and test validation is necessary prior to introducing this methodology into clinical practice, (iv) our study used measurement of serum cortisol by immunoassay rather than LCMS, and (v) we didn't measure serum levels of free cortisol or cortisone. However, our results relating the serum with salivary measurements fit with the previous literature, the strong correlation between free serum cortisol and salivary cortisol has been extensively reported [11,12], as has the lack of rise of serum free cortisone following synacthen [12]. The use of healthy volunteers has the advantage of a very tightly controlled study with a uniform population of subjects. Further work is required to demonstrate whether similar responses are seen using different assay platforms, in non-dexamethasone suppressed individuals and those with adrenocortical insufficiency.

Our results indicate that salivary cortisone reflects the serum cortisol response to synacthen but suggest that a 60 minute sample would be best used for diagnostic accuracy. However, before advocating replacement of a serum-based cortisol assay for cortisol quantification following synacthen administration, further studies are required in patient populations, without dexamethasone suppression and using LCMS in order to confirm our findings and define normal ranges of salivary cortisone and the diagnostic cutoffs for adrenal insufficiency.

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Table

Table 1. Median peak plasma cortisol and salivary cortisol and cortisone concentrations (Cmax) and time to Cmax (Tmax) following administration with low dose (1 mcg) and high dose (250 mcg) synacthen.

	Serum cortisol		**Salivary cortisol**		**Salivary cortisone**	
Synacthen dose	**1 mcg**	**250 mcg**	**1mcg**	**250 mcg**	**1 mcg**	**250 mcg**
Median Cmax (IQR) in mcg/dL (*nmol/L*)	14 (*386*) (12-16) (*333-430*)	22 (*615*) (21–24) (*583-647*)	0.3 (*7.3*) (0.2-0.5) (*5.1-12.9*)	1.2 (*32.9*) (1.0-1.3) (*28.6-35.0*)	1.1 (*31.4*) (0.9-1.6) (*24.4-42.7*)	2.8 (*75.9*) (2.6-3.0) (*71.8-82.0*)
Modal Tmax in minutes	30	120*	30	120*	40	120*

* final sampling time point – Cmax may be beyond this and therefore Tmax later.

Figure legends

Figure 1. Serum cortisol, salivary cortisol and salivary cortisone responses from baseline to 120 minutes following administration with low dose (1 mcg) and high dose (250 mcg) synacthen. Individual, mean and mean +/- 1 standard deviation shown.

Figure 2. (a), Scatter graph showing relation of serum cortisol to salivary cortisol by time after synacthen administration (intercept 2.17, slope 0.387). (b), Scatter graph showing relation of serum cortisol to salivary cortisone by time after synacthen administration. The mixed effects model line is shown in both (salivary cortisone intercept 1.23, slope 0.823), but for both salivary cortisol and cortisone the model does not include the time points between after time zero and before 60 minutes, as it is evident there is a lag in salivary response with these points falling above the model line.

Figure 3. Serum cortisol plotted against salivary cortisone at each time point from baseline (-1 minute) to 120 minutes following synacthen administration. The mixed effects model line is shown at each time point.

Figure 4. (a), Correlation coefficients of serum cortisol and salivary cortisol at each time point following synacthen administration (correlation coefficients for -1, 5 and 10 minute samples not significant). (b), Correlation coefficients of serum cortisol and salivary cortisone at each time point following synacthen administration.