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Cortisol in Saliva – Reference Ranges and Relation to Cortisol in Serum

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Summary: The aim of this study was to establish morning and evening reference ranges for cortisol in saliva. Another objective was to compare the concentrations of the mainly free cortisol in saliva to those of total cortisol in serum as determined with a commercial radioimmunoassay. The concentrations were determined in matched samples of saliva and serum collected at 8 am and 10 pm from 197 healthy volunteers. The saliva samples were stable for at least 7 days at room temperature and for 9 months at −20 °C. Reference ranges, the central 95%, were estimated to 3.5–27.0 nmol/l at 8 am and < 6.0 nmol/l at 10 pm. The intra-assay coefficient of variation (CV) was below 5% and total CV below 10%. The relation between the cortisol concentrations in serum and saliva was non-linear with r = 0.86 for serum concentrations < 450 nmol/l and r = 0.44 for serum concentrations ≥ 450 nmol/l. In conclusion, the satisfactory precision of the analysis and the simple non-invasive sampling procedure suggest that saliva may be used for cortisol measurements in situations where blood sampling is difficult to perform.

Introduction

Determination of cortisol in serum and urine has long been used in the assessment of adrenocortical function and other disturbances in the hypothalamic-pituitary-adrenocortical axis. It also serves in the diagnosis of depressive disorders (1, 2), and as the most frequently used marker for different kinds of stress-induced reactions (3–6). In serum, cortisol is mainly protein-bound and is usually measured as such. The analysis of free cortisol in serum is time-consuming and expensive and not suitable for clinical routine (7). In urine cortisol exists only in free form. Renal secretion, however, is dependent on glomerular and tubular function and the measured daily secretion rate depends on a correct 24-hour collection of urine. Hence the urinary cortisol does not always correctly reflect the free cortisol concentration in serum.

In serum, cortisol is to 90–95% bound to proteins, about 60% to transcortin and 30% to albumin. Transcortin binds cortisol with high affinity but low capacity, whereas albumin has a low affinity but an almost infinite capacity for cortisol. Transcortin is saturated at a serum cortisol concentration of 450–500 nmol/l (7, 8), thus already within the reference range at 8 am of 200–800 nmol/l. At levels exceeding transcortin saturation, the unbound fraction increases. In various clinical conditions the transcortin concentration and thereby the total serum cortisol concentration changes. Pregnancy and the use of oral contraceptives and oestrogens increase the transcortin concentration 2–3 times. Whether the free, biologically active fraction changes or not is at present a controversial issue (9, 10). In certain liver and kidney diseases and in various catabolic conditions the level of transcortin decreases, resulting in decreased serum cortisol concentrations, while the unbound fraction is said to remain constant (9).

In saliva, cortisol appears mainly in free form. Its concentration is approximately two thirds of unbound cortisol in serum and correlates well with this serum fraction (9). About 15% of salivary cortisol is bound to transcortin, a normal component of uncontaminated parotid saliva (11–13). The transfer from serum to saliva occurs by free diffusion of unbound cortisol through the acinar cells of the salivary glands (14, 15) and the equilibrium between serum and saliva is reached in less than 5 min-

utes (9). The salivary cortisol concentration is independent of saliva flow rate and of the serous and mucous content (16–18).

The above described disadvantages when measuring free cortisol in serum and urine are circumvented with the use of saliva. The aim of this study was therefore to evaluate our current cortisol method, used for serum and urine, for measurement of the cortisol concentrations in saliva and to establish its reference ranges. Also, by a simultaneous sampling of blood a correlation could be made with the cortisol concentration in serum.

Materials and Methods

Subjects

The study material comprised samples from 197 healthy individuals, 123 men (age range 20–70 years) and 74 women (age range 20–70 years). The material consisted of 66 men from the local rescue service and 57 men and 74 women randomly selected from the local population. The subjects were not taking any kind of steroid compound, except contraceptives (13 women). All subjects contributed at least one morning and one evening saliva sample during a 24-hour period, altogether 434 samples. Blood was also drawn, the venipuncture being performed after the saliva collection in order to avoid stress-induced increase in cortisol concentration. In ten of the subjects (4 men, 6 women), the intra-individual variation was determined on morning and evening samples collected within 24 hours on three occasions at one week's interval. In order to exclude possible variations related to leisure time, the samples were collected on Tuesdays, Wednesdays or Thursdays. The morning samples were collected at 8 am ± 45 minutes and the evening samples at 10 pm ± 45 minutes.

Procedure of saliva collection

To avoid an increase in cortisol concentration and contamination of the oral cavity due to food intake or smoking (19), the test subjects were instructed not to eat, drink or smoke later than 60 minutes before the samples were collected. The subjects were not allowed to expose themselves to physical strain later than 60 minutes before sampling and they were instructed to rest lying down during the last 30 minutes. Brushing of the teeth was not allowed during the 60 minutes preceding saliva collection to minimize the risk of blood contamination. The mouth was to be rinsed with water 15 minutes prior to saliva collection.

The saliva was collected in a Salivette® test tube (Sarstedt, Rommelsdorf, Nümbrecht, Germany) equipped with an insert containing a steril cotton-wool swab through which the saliva passes during centrifugation, yielding a saliva free from particles. The swab was drained with saliva during 60 seconds, as recommended by the manufacturer, and was then put back into the insert. Within this time, a few subjects did not produce enough saliva, i.e. 0.5 ml. Swabs treated with citric acid were tested for morning and evening samples from 5 individuals for comparison with plain swabs. None of the test subjects complained of any discomfort in connection with the saliva collection except for the bad taste of the citric acid treated swabs.

Preparation of samples

The samples were centrifuged at 1900 *g* for 15 minutes at room temperature and were then frozen at −20 °C within 4 hours after sample collection. The longest storage time was 9 months. Prior to analysis samples were thawed at room temperature and tested for blood contamination using a HemoCue® (HemoCue AB, Ängelholm, Sweden) microcuvette for haemoglobin determination. All samples from one individual were analysed in the same assay.

Method

Cortisol was measured by a solid phase radioimmunoassay, Coat-A-Count from Diagnostic Products Corporation (DPC), Los Angeles, USA. This kit is used in our laboratory for the analysis of cortisol in serum and urine. For serum our current reference range is 200–800 nmol/l at 8 am and < 300 nmol/l at 10 pm. For detection of the low concentrations present in saliva, the sensitivity of the method needed to be increased. Following the instructions of the manufacturer the sample volume was therefore increased from 25 µl to 200 µl and the incubation time extended from 45 minutes at 37 °C to 3 hours at room temperature. Calibrators and controls (DPC) were also adapted to saliva analysis by dilution in water 1 : 10. The measurable range was 0.5–138 nmol/l. Quality controls of three different concentrations (2.3, 12.6 and 123 nmol/l) were processed at the beginning and at the end of each assay. In assays of more than 50 samples, controls were also processed in the middle of the assay.

Statistics

Statistical analyses were made using *Mann-Whitney*'s non-parametric test for unpaired measurements (20).

Results

The detection limit of the method was assessed by analysing the zero calibrator in duplicate in eighteen different assays. Defined as apparent concentration 2 standard deviations below mean counts per minute for the zero calibrator, the detection limit was 0.5 nmol/l.

The precision was examined by analysing controls, one sample with high and one sample with low cortisol concentration in duplicate in eight different assays. These samples were frozen in aliquots to avoid refreezing. The coefficients of variation (CV) were calculated from the means of the duplicates. The intra-assay CV was 4.3% for values 0.5–10.0 nmol/l and 3.6% for values 10.1–123 nmol/l. Total CV, intra- and inter-assay CV, was 8.3% for values 0.5–10.0 nmol/l and 5.1% for values 10.1–123 nmol/l. On five occasions these samples were analysed at the beginning and at the end of an assay of more than 300 tubes without showing any drift of the measured cortisol concentration. For the serum samples in this study the total CV, determined from ten means of duplicates of controls, was 7.9% and 4.4% for concentrations of 310 and 850 nmol/l, respectively.

The accuracy of the method was assessed by recovery experiments where samples from four subjects were spiked with calibrators of three different concentrations. There was a good agreement between expected and observed results (tab. 1). The stability of the saliva samples was tested by analysing 3 samples with different cortisol concentrations stored under different conditions (tab. 2). The samples were stable at room temperature for at least

7 days and were also unaffected by freezing at −20 °C for up to 9 months. After refreezing, however, the cortisol concentrations showed a marked decrease. Use of citric acid treated swabs resulted in 10−70% higher concentrations.

Reference ranges for salivary cortisol were established from 195 morning and 195 evening samples (tab. 3).

Tab. 1 Recovery experiments. Samples from four different subjects spiked in dilution 1 : 2 with calibrators of different concentrations.

Sample	Cortisol (nmol/l)			Observed
	Calibrator	Expected	Observed	Expected
5.0	138.0	71.5	71.4	1.00
7.4	2.8	5.2	5.8	1.11
7.4	27.8	17.6	18.4	1.04
16.0	138.0	77.0	74.6	0.97
21.0	138.0	79.5	74.0	0.94

Women under the age of 40 had significantly higher cortisol concentrations than women over 40. The 12 women, 21−40 years old, who were taking oral contraceptives (the 13th woman was over 40 years) had a mean concentration of 23 nmol/l at 8 am and of 2.2 nmol/l at 10 pm. The 42 untreated women in the same age groups had a mean concentration of 11.5 nmol/l at 8 am and of 1.1 nmol/l at 10 pm. Beyond the age of 40 the cortisol concentrations appeared to decrease with increasing age, but the differences were without statistical significance. The reference ranges, defined as the central 95%, were estimated to 3.5−27.0 nmol/l at 8 am and < 6.0 nmol/l at 10 pm for both sexes including all ages, and for practical reasons also the women on contraceptives. In 23 (11%) of the evening samples the cortisol concentration was below the detection limit of the method.

The correlation between the cortisol concentrations in serum and saliva is presented in figure 1. The relation-

Tab. 2 Stability of three saliva samples stored under different conditions.

Storage	Time (days)	Salivary cortisol (nmol/l)		
		Sample 1	Sample 2	Sample 3
Analysed immediately	–	2.7	7.1	18.9
Room temperature	7	2.8	6.9	19.0
Refrigerator (2−8 °C)	7	2.7	7.1	18.7
Freezer (−20 °C)	30	2.7	7.0	18.9
Freezer (−20 °C)	270	2.6	7.2	19.6
Refrozen (−20 °C) after thawing	7 + 23	2.3	6.6	18.3

Tab. 3 Salivary cortisol concentrations (nmol/l) of the reference population.

Age (years)	Sex	N	Cortisol (nmol/l)			
			Morning		Evening	
			Mean	Range	Mean	Range
21−30	♂	26	10.0	3.1−20.5	2.2	<0.5−8.5
	♀	20	14.6*	7.5−37.2	1.1	<0.5−2.9
31−40	♂	37	12.1	3.3−42.8	1.4	<0.5−9.9
	♀	22	15.0*	2.6−41.8	1.7	<0.5−3.7
41−50	♂	30	11.4	4.9−30.0	1.6	<0.5−9.1
	♀	9	11.4	3.0−35.7	2.2	0.7−5.0
51−60	♂	21	10.3	3.1−22.4	1.6	<0.5−6.3
	♀	13	10.0	4.1−20.4	2.6	0.6−7.0
61−70	♂	7	9.7	4.7−18.2	2.1	0.7−3.7
	♀	10	9.1	7.3−15.7	1.5	0.6−3.1
Total	♂	121	10.7	3.1−42.8	1.8	<0.5−9.9
	♀	74	12.0	2.6−41.8	1.8	<0.5−7.0
		195	11.9	2.6−42.8	1.8	<0.5−9.9

* $p < 0.005$ vs women over 40 years.

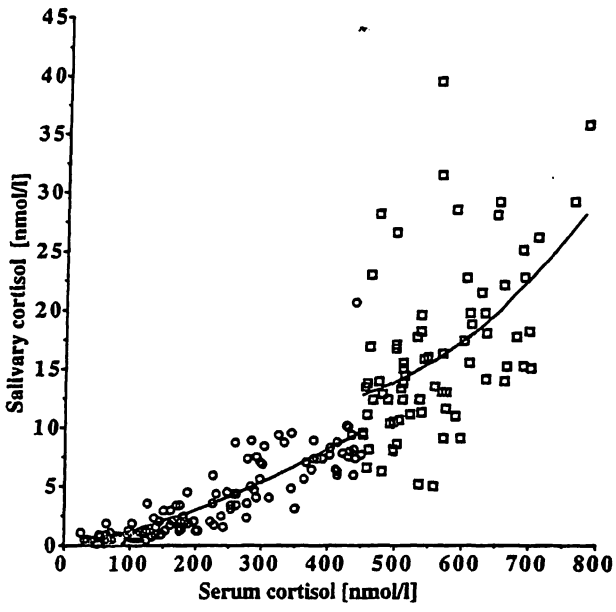

Fig. 1 Correlation between the concentrations of cortisol in saliva and serum.
Serum concentrations < 450 nmol/l (○) n = 244.
Correlation with saliva concentrations r = 0.86 ($p < 0.0001$).
Serum concentrations ≥ 450 nmol/l (□) n = 146.
Correlation with saliva concentrations r = 0.44 ($p < 0.0001$).
○ $y = -0.33 + 0.01x + 2.21 \times 10^{-5} x^2$
□ $y = 22.33 - 0.06x + 8.71 \times 10^{-5} x^2$

ship was non-linear with r = 0.86 for serum concentrations < 450 nmol/l and r = 0.44 for serum concentrations ≥ 450 nmol/l. With serum concentrations above 450 nmol/l the ratio of saliva to serum concentration was increased from 2.1% to 3.0%. Intra-individual variations in salivary cortisol concentrations and its relationship to serum cortisol are shown in figure 2.

The effect of blood contamination was tested by adding increasing amounts of blood to a saliva sample with a cortisol concentration of 7.4 nmol/l. At a haemoglobin concentration of 2 g/l a pellet of erythrocytes was visible after centrifugation but this small contamination did not increase the cortisol level. However, at a haemoglobin concentration of 5 g/l, the cortisol level was increased by 5%, at 8 g/l by 14% and at 12 g/l by 30%. In the reference material were included 26 samples with a haemoglobin concentration of 1–2 g/l since this small contamination had no effect on the salivary cortisol concentration. Two samples were excluded from the reference material due to haemoglobin concentrations of 12 g/l and 16 g/l, respectively.

Discussion

The mean salivary cortisol concentrations determined with the Coat-A-Count kit (DPC), 11.9 nmol/l at 8 am and 1.8 nmol/l at 10 pm, may be compared to values obtained with other radioimmunoassays, i. e. a mean of 15.5 nmol/l at 8 am and 3.9 nmol/l at 8 pm (21) and a median of approximately 11 nmol/l at 9–10.30 am (22). Such minor differences in the results are to be expected considering the difference in method, population and sampling time. Alone the use of different radioimmunoassays may give concentration differences of 220% (23). Nevertheless, the reference range established in the current study, 3.5–27 nmol/l at 8 am and < 6 nmol/l at 10 pm, does not differ much from the one earlier report of a reference range defined as the central 95%, i. e. 5–28 nmol/l (22). A reference range for evening cortisol concentrations was not given in this latter report. Other studies reporting on mean cortisol values, which abound in the literature, were either based on insufficiently defined populations as to age or sex distribution, or comprised relatively small numbers of subjects, at the most a few dozens (24). They were therefore apparently not intended for the establishment of reference ranges which demands well over 100 samples (25). Neither can a reference range be calculated from the occasionally given mean and standard deviation since the distribution of cortisol concentration is not *Gaussian* (own observation).

The higher cortisol concentrations in morning saliva in women under the age of 40 can be explained by their use of oral contraceptives (26, 27). Studies have revealed elevated salivary cortisol during the day in late pregnancy and after long-time use of oral contraceptives (9, 10). The cortisol in saliva being mainly free, implying that the elevation of the salivary cortisol concentration is not due to higher concentrations of transcortin. Many possible causes of the increased concentration of free cortisol have been suggested, one being an altered sensitivity of the hypothalamic-pituitary-adrenal axis by increased oestrogen levels (28). The slight decrease in morning cortisol concentrations with increasing age could be caused by a reduction in the transport of cortisol from blood to saliva due to physiological changes in old age (29).

When comparing salivary and serum cortisol concentrations, high correlations have been found with r = 0.71 to r = 0.96 (30). In this study a similar high correlation, r = 0.86, is seen only with serum concentrations < 450 nmol/l. The relation of salivary to serum cortisol concentration agrees well with theoretical considerations. Salivary cortisol has proved to be only about 70% of the free cortisol concentration in serum, probably due to the presence of 11β-hydroxysteroid-dehydrogenase in the salivary gland (16). At total serum cortisol concentrations of about 450 nmol/l with 3–5% in unbound form (7), the cortisol concentration in saliva would correspond to approximately 2.1–3.5% of total serum cortisol. In this study the concentration in saliva was on average 2.1% of serum concentrations < 450 nmol/l and 3.0% of serum concentrations ≥ 450 nmol/l.

As shown in figure 2, the relationship between individual values of cortisol in saliva and serum remained fairly constant in the 3 morning as well as the 3 evening samples. The 10 subjects apparently present individual patterns which may be due to different activities of the 11β-hydroxysteroid-dehydrogenase. Serum concentrations exceeding 450 nmol/l were in most cases accompanied by a marked increase in salivary cortisol.

Blood contamination of the saliva may cause spuriously high cortisol values. The risk of blood contamination is evident in patients suffering from gingivitis. In this study contamination occurred in 28 (6%) of the samples. However, only 2 of these were contaminated by blood in amounts that affected the result. To avoid this error, we propose that saliva samples with a visible erythrocyte pellet be analysed for haemoglobin and a new sampling made when the concentration is over 5 g/l.

An advantage of saliva over serum cortisol determination is the simple sampling procedure and thereby the avoidance of a stress-induced rise in cortisol concentration. Further, the sample can be taken by the patient at home and sent by mail to the laboratory for analysis. Transportation at room temperature is possible thanks to the stability of cortisol in saliva. The stability can be

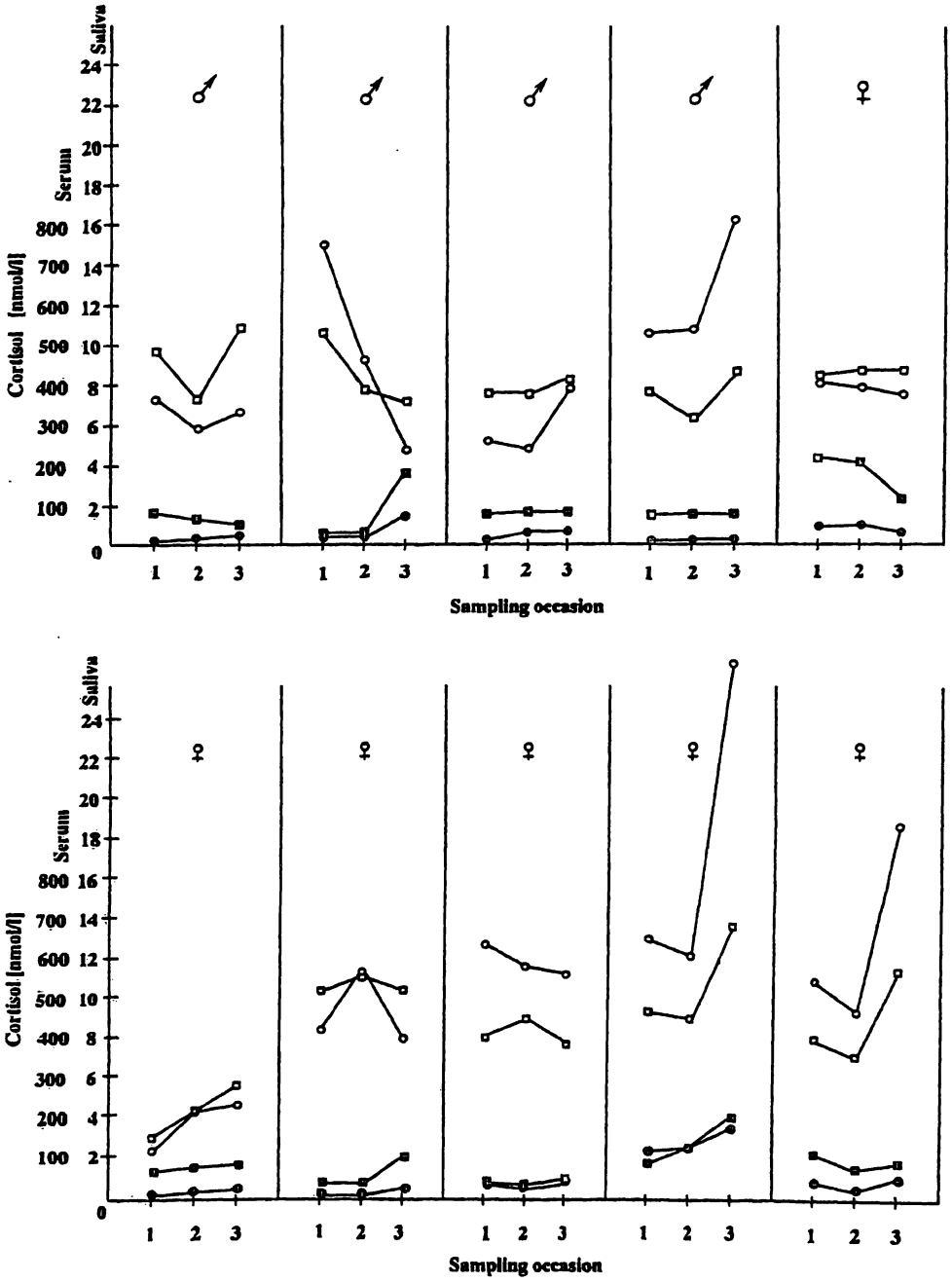

Fig. 2 Individual values of cortisol concentrations in saliva and serum in 10 subjects (4 men, 6 women). Morning and evening samples were collected within a 24-hour period on three occasions at a week's interval.

Salivary cortisol concentration: Morning sample (○), evening sample (●).
Serum cortisol concentration: Morning sample (□), evening sample (■).

further enhanced by the use of Salivette® tubes with citric acid treated swabs (31), but when measured with the DPC kit the cortisol concentration will then be higher in most samples. Sampling of saliva is therefore preferable under extreme conditions when transportation to the laboratory is delayed or when refrigeration facilities are not available (31). These advantages are of value in disaster research, where cortisol can be used as a marker for stress (5, 32). It is also useful in patients on whom venipuncture is difficult to perform and when repeated sampling is needed as in the assessment of hypothalamic-pituitary-adrenocortical function (7, 33–35).

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