Growth hormone and cortisol in serum and saliva

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Rantonen PJF, Penttilä I, Meurman JH, Savolainen K, Närvänen S, Helenius T. Growth hormone and cortisol in serum and saliva. Acta Odontol Scand 2000;58:299–303. Oslo. ISSN 0001-6357.

Salivary diagnosis is a developing area in clinical chemistry and dentistry. Cortisol analyses from saliva have been used in pediatric practice and as doping tests. Growth hormone (hGH), also a stress hormone, has not been analyzed from saliva. We studied the serum and saliva of 51 healthy subjects. The samples were taken at 8:00 in the morning after 12 h fasting. Cortisol concentrations were analyzed using RIA. An immunoradiometric assay was applied for analyzing serum and salivary hGH. The validity of this method developed in our laboratory was found to be good. The results showed correlation of salivary cortisol with that of serum ($r = 0.47$, $P < 0.001$). Salivary hGH concentrations were 1000-fold lower than the respective values in serum, but a clear correlation was found between salivary and serum hGH levels ($r = 0.59$, $P < 0.001$). □ *cortisol; diagnosis; growth hormone; metabolism; saliva; serum*

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Salivary diagnosis is an increasingly interesting field in dentistry, internal medicine, endocrinology, pediatrics, immunology, clinical pathology, psychology, and sports medicine (1). A growing number of drugs, hormones, and antibodies can be monitored in saliva, which is an easily obtainable, non-invasive diagnostic medium (2–4). Salivary diagnosis is therefore suggested as being particularly useful in cases where repeated samples of body fluid are needed but where drawing blood is impractical, unethical, or both. In addition, salivary concentrations of drugs and hormones represent the free fractions of serum in many instances, with good correlations with the respective total concentrations in serum (5).

The present study was done to investigate salivary concentrations of cortisol and human growth hormone (hGH, somatotropin) in a healthy homogeneous study group. The two hormones where monitored because previous studies on the metabolism of fasting have shown that concentrations of these stress hormones in blood increase during fasting (6, 7). This may partly explain the subjective symptoms experienced during fasting (8). Cortisol stimulates lipolysis by increasing cAMP concentration during fast (9). Also growth hormone stimulates lipolytic activity and ketogenesis during fasting (10, 11). Salivary diagnosis for analyzing the concentrations of these hormones would provide an obvious benefit over repeated blood samples. To our knowledge, there are no earlier reports of growth hormone having been analyzed from human saliva. Cortisol, on the other hand, has been successfully analyzed in saliva (5). Our study hypothesis was that salivary concentration of hGH would correlate with its respective serum concentrations, that is, if hGH in general was detected in saliva. Because gender is the important variable in hormone studies, we tested it as the independent variable. Cortisol and salivary total protein were analyzed for reference.

Subjects and methods

Subjects

Fifty-one healthy voluntary subjects (23 men, 28 women) participated in the study. The inclusion criteria did not allow any medication, including female sex hormones, or smoking. The subjects' mean age was 23.7 years (SD 2.5; range, men 20.5–30.0, range, women 20.5–28.0). Information about their health was recorded during an interview before collecting the first salivary samples in the morning. There was no difference in this between the genders. The study protocol had been accepted by the Ethics Committee of Kuopio University Hospital.

Blood and saliva samples

The subjects were given written instructions beforehand regarding the saliva and serum collections. Saliva samples, taken before the blood samplings, were always taken at the same time in the morning (at 8:00). The subjects were told not to eat or smoke for 12 h before the sampling.

The subject was seated comfortably, with the eyes open. Unstimulated saliva was collected for a single 5-min period with the subject leaning forward and letting the saliva drain into a graded sampling tube. Before collection, the mouth was emptied by an initial swallow. Stimulated whole saliva was collected over a 3-min period, and the saliva secreted during the first 30 s was discarded. Saliva flow was stimulated by chewing a 1 g piece of paraffin wax

(Orion Diagnostica, Espoo, Finland) at a constant rate (about once a second). The samples were then centrifuged (10 min, 2000 × *g*) and deep-frozen (−70°C) for later analyses. Salivary total protein was then analyzed using the Lowry method (12).

For salivary growth hormone analysis, each participant gave saliva samples directly into albumin-coated test tubes that were kept in crushed ice. Immediately after collection, the samples were deep-frozen in dry ice and stored at −70°C until analyzed.

Cubital venous blood was drawn into 5 ml Vacutainer tubes without anticoagulants. Coagulated blood was centrifuged (10 min, 720 × *g*), serum was taken and deep-frozen (−70°C) for analyses.

Analyses of serum and salivary cortisol concentrations

An Orion Diagnostica RIA kit was used (Orion Diagnostica, Espoo, Finland) to analyze serum cortisol. In brief, lyophilized cortisol antiserum was diluted in 11 ml of assay buffer. Lyophilized standards in cortisol-free human serum (0–2000 nmol/l) were diluted in 1 ml of distilled water. Then 25 µl aliquots of the standards, controls, and study samples were pipetted into test tubes, 0-standard was pipetted into the non-specific binding (NSB) tube; 100 µl of ^{125}I-cortisol solution was then added to each test tube and 100 µl of antiserum solution added to all except the total activity and NSB tubes. Only 100 µl of assay buffer was added to the NSB tubes. The tubes were then shaken in a Vortex mixer, covered with a paraffin foil, and incubated at 37°C for 60 min. After 10 min at room temperature (20°C) 1 ml of polyethylene glycol solution was added, and the tubes were mixed in the Vortex mixer. After 15 min centrifugation (2000 × *g*) the supernatants were discarded, the tubes were wiped dry and radioactivities assessed using a Gamma Master 1277 gamma-counter (Wallac Oy, Turku, Finland) during 1 min. All the analyses were made in duplicate. The intra-assay CV of the method is 1.7–4.1% (range 180–1200 nmol/l) and the inter-assay CV 5.2–9.6% (range 31.2–1090 nmol/l). The recovery of added cortisol is 101%.

Salivary cortisol was assessed similarly as from blood samples, except that the standards were prepared from a 2000 nmol/l solution which was diluted in the 0.1 M Tris buffer (pH 7.4) to give final concentrations of 0–100 nmol/l. The controls were also diluted to fit the standard line drawn. Antiserum and assay buffer for the NSB tubes were diluted 1:5 in the Tris buffer. Standards, controls, and saliva samples were all taken in 100 µl aliquots for the analyses. The salivary analyses, too, were made in duplicate. According to the manufacturer of the analysis kit, the intra-assay CV of the method is 4.4% in the concentration range 8–57 nmol/l.

Assay for human growth hormone in saliva and in serum and its validation

The concentrations of hGH in saliva and in serum were determined with a highly sensitive immunoradiometric assay, which originally was introduced for urine samples (13). Two monoclonal antibodies were used, both from Medix Biochemica (Kauniainen, Finland). Mab 5802 (K = 1 × 10E10) coated to Maxisorp tube (Nunc, Denmark) was used as a catching antibody and Mab 5801 (K = 3 × 10E10) was iodinated with the chloramine-T method. The assay was calibrated against the WHO First International Reference Preparation 80/505 diluted in phosphate buffered saline (0.1 mol/l, pH 6.0) containing 1% bovine serum albumin. The assay was based on a two-step procedure. In saliva the procedure was as follows: first, 250 µl of centrifuged salivary sample or hGH standard was incubated in a coated tube for a minimum of 6 h at 4–8°C. After two washings the labelled antibody was allowed to react with bound hGH overnight at 4–8°C. The tube was washed four times and its radioactivity was counted. In serum, the procedure was the same, but 50 µl of serum and 200 µl of phosphate buffered saline (0.1 mol/l, pH 6.0) were used.

According to Medix Biochemica, both monoclonal antibodies recognize specifically human growth hormone with molecular weight of 22 kDa (cross-reactivity 100%). Neither of the monoclonal antibodies recognizes the molecular form of 20 kDa (cross-reactivities below 0.2%). Cross-reactivity with human prolactin in 1% with both antibodies. With human placental lactogen, the Mab 5801 has a cross-reactivity of 0.2% and Mab 5802 below 0.2%. The detection limit of the assay, estimated from the interpolated response at two standard deviations above zero dose, is 1.0 ng/l when fresh labelled antibody is used.

The inter- and intra-assay CVs are 5–10% throughout the measuring range. The inter-assay CVs for urine are 13% and 4.6% at the levels of 6 ng/l and 51 ng/l, respectively (Medix Biochemica).

To test the linearity of the method, we assayed serial dilutions with zero standard of two such salivary samples in which blood contamination could not be shown. Dilutions of 1:2, 1:4, 1:8, and 1:16 were done. The results for the first sample were 69, 35, 16, and 9 ng/l, respectively, and for the second sample 40, 20, 10, and 6 ng/l, respectively. The mean analytical recovery of hGH added to five different salivary samples was 102.8% (range 96–112%).

The possible blood contamination of the salivary samples was tested with semiquantitative strip-test (Rediatest, Boehringer Mannheim, Germany). The validity of the test for salivary samples was verified by making dilutions of blood with saliva. The sensitivity of the assay is 1 × 10 E 7 erythrocytes/liter. The range of erythrocytes was <1 × 10 E 7–5 × 10 E 9/1. Because the normal range of erythrocytes in blood is 4–6 × 10 E 12/1 only in two cases, part of the measured concentration of hGH in saliva was derived from blood contamination.

Analyses of serum growth hormone concentrations using radioimmunological method

Serum growth hormone concentrations were also

Table 1. Basic data of salivary and serum samplings ($n = 51$)

	Women		Men		All	
Parameter	Mean	SD	Mean	SD	Mean	SD
Saliva						
Unstimulated flow (ml/min)	0.5	0.3	0.5	0.2	0.5	0.3
Stimulated flow (ml/min)	1.5	0.6	1.6	0.5	1.5	0.6
Protein (mg/ml)	0.8	0.3	0.8	0.2	0.8	0.8
Cortisol (nmol/l)						
Unstimulated	20	5.6	25.1	23.6	22.3	16.4
Stimulated	19.5	5.5	20.8	14.5	20	10.4
hGH (mU/l)	12.8	13.5	3.4	2.3	8.6**	11.1
Serum						
Cortisol (nmol/l)	809.5	261.4	514.1	139.6	676.3***	259.8
hGH (mU/l)	27.4	25.6	2.9	9.4	16.4***	23.3
Radioimmunological method						
hGH (μg/l)	13.7	9.3	1.5	2.3	8.2***	9.3

Difference between genders: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

analyzed using the radioimmunological method of Orion Diagnostica Human Growth Hormone RIA kit (Espoo, Finland). Lyophilized standards in bovine serum (0–62.5 ?g/l) were diluted in 1.0 ml of distilled water. The standards have been calibrated against the WHO First International Reference Preparation (WHO IRP 66/217) of human growth hormone. The rabbit hGH antiserum mainly recognizes the 22 kDa isoform. The 20 kDa isoform cross-reacts under 10%. Aliquots of 100 μl of the standards, controls, and serum samples were pipetted into the coated RIA tubes. 200 μl ^{125}I-labelled hGH and 200 μl antiserum were added to the mixtures. The tubes were covered with paraffin foil and incubated overnight at 20°C. After decanting, the tubes were washed with 1.0 ml of washing solution. Thereafter, radioactivity was assessed in a Gamma Master 1277 gammacounter for 1 min. All the analyses were done in duplicate. The intra-assay CV of the serum GH method is 6.1–1.9% in the concentration range 1.4–12.6 μg/ml and the inter-assay CV 13.7–4.0% in the range 1.4–12.6 μg/ml. The recovery of added GH is 101%.

Statistics

Mean concentrations and standard deviations of variables were calculated. Differences between genders were calculated using Student's *t*-test for independent samples. Kolmogorov-Smirnov's test was used to test the normality of distributions of cortisol and growth hormone data. To remove skewness in these values, concentrations were re-expressed as base 10 logs. In addition, concentrations of salivary proteins can be subject to variation due to differences between the subjects in flow rates and total protein output (14). We used Pearson's partial correlation controlling for salivary total protein to calculate associations between variables.

Results

Table 1 gives the basic characteristics of the salivary and serum samples. Salivary urea level was significantly higher in men than in women ($P < 0.05$). Salivary growth hormone level was significantly higher in women than in men ($P < 0.01$). Serum cortisol and growth hormone levels were significantly higher in women than in men ($P < 0.001$).

Distributions of cortisol and growth hormone scores were substantially skewed, as is often the case with salivary data. To remove skewness in these values, concentrations were re-expressed as base 10 logs (Table 2).

Partial correlations were calculated between serum and saliva levels of these hormones. Salivary cortisol was significantly associated with serum cortisol level ($r = 0.47$, $P < 0.001$) (Fig. 1). Salivary and serum growth hormone levels also correlated significantly with each other ($r = 0.59$, $P < 0.001$; men $R = 0.27$, n.s; women $R = 0.44$, $P < 0.05$) (Fig. 2). The two different methods for analyzing human growth hormone concentrations correlated significantly with each other ($r = 0.90$, $P < 0.001$).

Partial correlations between salivary and serum cortisol and growth hormone levels and total protein were also

Table 2. Skewness values for cortisol and growth hormone in serum and saliva

	Mean	Skewness	Log[a]	Skewness
Saliva				
Cortisol (nmol/l)	20	3.2	1.3	0.3
hGH (μU/l)	8.6	2.8	0.7	0.8
Serum				
Cortisol (nmol/l)	676.3	0.5	2.8	0.1
hGH (mU/l)	16.4	2	0.5	0.3

[a] Summary statistics computed from original values re-expressed as base 10 logarithms.

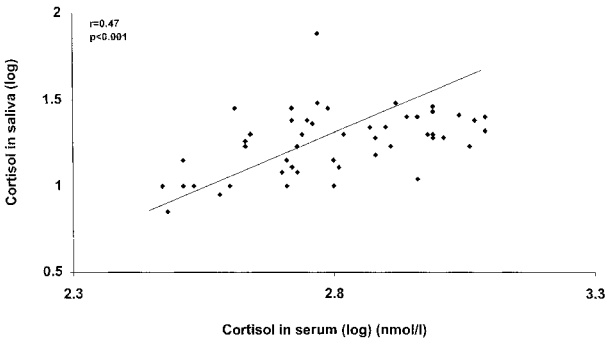

Fig. 1. Correlation between serum and stimulated salivary cortisol levels in 51 healthy subjects after 12-h fasting.

calculated. Salivary hormone levels did not correlate with total protein variables.

Discussion

This study was the first to assess growth hormone in human saliva. This peptide hormone contains 191 aminoacids and it is secreted in pulses from the anterior part of the pituitary gland (15). Its function in the oral cavity is not known. The secretion from the pituitary gland follows a diurnal rhythm, with a peak early in the morning. It is therefore pertinent to take samples for hGH assessments always at the same time of the day, as we did in the present study. Because only minor amounts of hGH were detected in saliva, it can be anticipated that it is not actively excreted into saliva, but that salivary hGH rather represents serum ultrafiltrate and is probably part of the free fraction of plasma, devoid of any binding globulin. Further, hGH is a large molecule (molecular weight 21.500) and it is not probable that it can diffuse freely from serum to saliva as steroid hormones, for example, whose size is much smaller (5). The tiny amounts of hGH detected in saliva also disprove any particular active transport system within the salivary glands and ducts. But we cannot exclude the possibility that hGH might still be excreted into the salivary gland secretions because we sampled whole saliva, or rather oral fluid, which is known to contain a variety of degradative enzymes and microbial products which may degrade hGH molecules also. Thus, further studies are needed to explain the secretion and eventual function of hGH in saliva. However, we found clear correlation between serum and salivary hGH levels, which is a rather surprising result considering the size of the hGH molecule. It should also be noted that the hGH concentrations detected in saliva were significantly higher in women than in men, thus reflecting the respective differences in serum concentrations between genders.

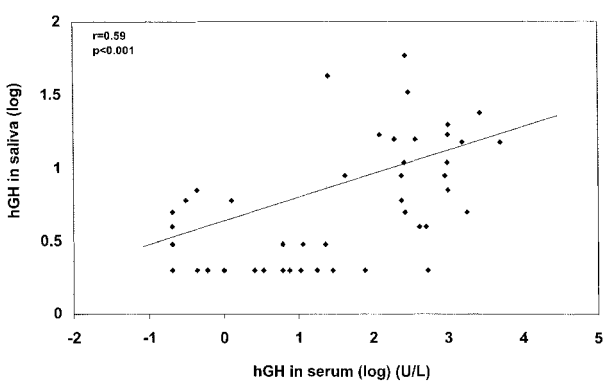

Fig. 2. Correlation between serum and unstimulated salivary hGH levels in 51 healthy subjects after 12-h fasting.

Routine methods used in clinical chemistry for the assessment of hGH, such as the RIA method used in the present study for serum samples, were not directly applicable for detection of the tiny amounts of hGH in saliva. Therefore we used an immunoradiometric assay originally developed for urine samples. Because it is highly important to use identical methods for both fluids, serum hGH concentrations were also measured by the immunoradiometric method.

There are only a few published reports on hGH and saliva. Baum et al. (16) found that hGH was secreted primarily in an exocrine manner into saliva after a parasympathomimetic secretory stimulus in rat. Alexander et al. (17) found that transgenic mice began to secrete hGH into saliva at puberty. On the other hand, it is known that saliva contains insulin-like growth factor I (IGF-1) and salivary IGF-1 levels reflect the GH status of the donor (18, 19).

Cortisol has been assessed in saliva also earlier (20–22). Salivary cortisol concentrations have been stated to be about 20–30% lower than free cortisol levels in plasma. Some of its metabolism has been anticipated to take place within the salivary glands (5). Cortisol, which is a glucocorticoid secreted by the adrenal cortex, is a well-known stress hormone with many functions, such as the regulation of cAMP (10). Its specific functions in the oral cavity, however, are not known. Salivary cortisol determinations have been used previously to elucidate its role in stress (23, 24). Also cortisol secretion follows a diurnal rhythm with peak concentrations in the morning, which emphasizes the need to standardize the time of samplings.

The previously published correlation coefficients for serum versus salivary cortisol concentrations range from $r = 0.86$ to $r = 0.97$ (20–23). Thus, our present material fell far behind the optimum in this respect (with the present $r = 0.47$). This was astonishing, because our test group was a homogeneous one with members being healthy students. Their salivary total protein concentrations, on the other hand, fell within the reference limits of our laboratory.

In conclusion, the present study showed that hGH was detected in saliva in tiny amounts, about 1000-fold less than in serum. However, there was a linear correlation

between salivary and serum hGH concentrations. Salivary cortisol concentration also showed a linear correlation with that in serum taken from the same subjects at the same time. However, these correlations are very low and neither growth hormone nor cortisol concentration in plasma could be inferred from salivary measurements. Further studies are called for to assess the role and function of these hormones in the oral cavity. Taking into account the very low concentrations of these hormones detected in saliva, it is probable that salivary concentrations indeed reflect passive diffusion rather than active secretion to the mouth. Therefore, it is anticipated that aspects of oral health, such as the degree of periodontal and mucosal inflammation, may affect the concentrations of cortisol and hGH detectable in saliva. Subsequently, further studies are needed in this respect, too.

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Received for publication 15 March 2000
Accepted 9 October 2000