CLINICAL APPLICATIONS OF SALIVARY CORTISOL MEASUREMENTS

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ABSTRACT

The clinical applications of salivary cortisol measurements were evaluated by radioimmunoassay of time-matched saliva and plasma samples. Salivary cortisol levels of normal subjects exhibited a significant ($p<0.001$) diurnal variation with a mean (±SD) concentration of 8.7 ± 4.8 nmol/L at 0800-1000 h and 2.4 ± 1.1 nmol/l at 1500-1700 h. After an overnight dexamethasone suppression test, morning salivary cortisol levels decrease to 2.7 ± 0.7 nmol/L ($p<0.001$ vs normal). An excellent correlation ($r=0.805$) of cortisol measurements with time-matched saliva and plasma samples was obtained ($y = 0.03x + 0.88$, $p<0.001$, $n=91$). Hypercortisolism was confirmed by raised salivary cortisols in only half of patients with elevated total plasma levels, thereby indicating that salivary cortisol measurements is a better index of adrenal status.

Keywords: Free steroid, adrenal status, salivary cortisol, total plasma cortisol

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INTRODUCTION

It is now recognised that the measurement of the free fraction of thyroid and steroid hormones in plasma provide the best indicator of hormone activity since it represents the biologically active form of the circulating hormone and is not dependent on alterations of binding globulin[1]. The methods available for determination of plasma free steroids include the technically complex procedures of ultrafiltration or equilibrium dialysis of plasma samples which are not suitable for routine use, or the cumbersome collection of 24-h urine. Since techniques similar to plasma free thyroid hormone assays[1] have not been developed for steroid hormones, the use of saliva samples have been introduced more recently[2-4].

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The measurement of salivary concentrations of adrenal [5-12], gonadal[13-15] and placental[16] steroids have been proven to be valuable clinical tools since their values reflect the free fraction in plasma. The additional advantages of salivary steroid assays are simple and easy collection of specimens which is a non-invasive and stress-free technique, multiple sampling and omission of the serum separation step[2-4]. It has been shown that salivary progesterone levels provide a useful index of corpus luteum function[14, 15] while salivary oestriol measurements[16] can be used for assessment of fetoplacental function. Home monitoring of efficacy of glucocorticoid replacement therapy in congenital adrenal hyperplasia (CAH) patients has been made possible through measurements of 17-alpha-hydroxyprogesterone and/or androstenedione levels of multiple saliva samples collected by patients themselves[10-12]. The use of saliva samples has also eliminated the requirement for frequent and often stressful venepuncture in CAH children. The technique can be also applied to neonates[17]. Although salivary cortisol levels may be up to 35% lower than the actual free fraction in plasma[6, 8], salivary cortisol concentrations are highly correlated with free plasma concentrations. The clinical usefulness of salivary cortisol assays have been proven by demonstration of circadian rhythm, and results from dynamic testing such as dexamethasone suppression and ACTH stimulation in normal subjects and in patients with Cushing's syndrome and adrenal insufficiency[5-9]. Since the salivary cortisol assay has been proposed as the method of choice for assessing adrenocortical function, we investigated further its clinical applications with our highly specific and sensitive in-house cortisol radioimmunoassay.

METHODS

Time-matched samples of plasma and saliva were collected and stored at -20°C until analysis. Venous blood samples of about 5 ml were collected by venepuncture from the forearm vein and put into heparinised tubes while 2-3 ml saliva samples were collected by direct salivation into a wide-mouthed vessel. We studied 108 (male = 66, female = 48; age range 17-45 yr) normal adult volunteers comprising staff and students of the Medical Faculty, Universiti Kebangsaan Malaysia (UKM), 11 of whom were given 50 mg cortisone acetate while 9 were given 2 mg dexamethasone for a period of three days. Samples were also obtained from 10 normal pregnant women in second trimester, 20 thyroid, 12 hypertensive and 9 diabetic patients from the Endocrine and Specialist Clinic, UKM.

An in-house radioimmunoassay was set up using

[1,2,6,7,-3H] Cortisol as tracer (Amersham Laboratories, England), preparations of cortisol (Sigma Chemical Company, USA) standards calibrated against WHO standards and dextran-coated charcoal for the separation of antibody bound and free fractions. All measurements were performed in duplicate. Standards and samples were pre-diluted in assay buffer 0.025 M citrate-phosphate, pH 5.1, at dilutions of 1/40 and 2/5 respectively for plasma and saliva assays. The antiserum was raised in rabbits against the antigen cortisol-3-0(carboxymethyl)-oxime bovine serum albumin (Sigma) and was used in final titre of 1/440. The major cross-reacting steroids were 17-alpha-hydroxyprogesterone 8.5%, prednisone 8.0%, corticosterone 0.3%, cortisone 0.25% and dexamethasone 0.08%. The minimal detectable concentration or sensitivity of the cortisol assay was 0.02 pmol/tube or 1.5 nmol/L; recovery of cortisol added to stripped plasma was 98 - 115% and recovery from saliva was 93 - 99%. The intra-assay coefficients of variation (cv) were 3.1 - 7.8% for cortisol levels ranging from 5.8-58 nmol/L while the corresponding inter-assay cvs were 7.5 - 10.4%. The in-house cortisol RIA was further validated by its performance in the WHO External Quality Control Scheme (EQAS) which gave a mean bias +2.7% and variance of 6.7% (n=21).

Statistical analysis was performed using Chi-square goodness-of-fit test to assess the normality of distribution and Student's unpaired t test. Linear regression analysis was used to assess correlation between salivary and plasma cortisol levels. A p value <0.05 was considered significant.

RESULTS

The circadian variations of cortisol levels in plasma and saliva of normal subjects are shown in Fig 1 and summarised in the Table I. There was a wide spread of individual data for each time point for total plasma cortisol concentrations as compared

Fig 1 - Diurnal variation of cortisol levels in time-matched plasma and saliva samples (n=108) collected from normal subjects from 0800 to 1700

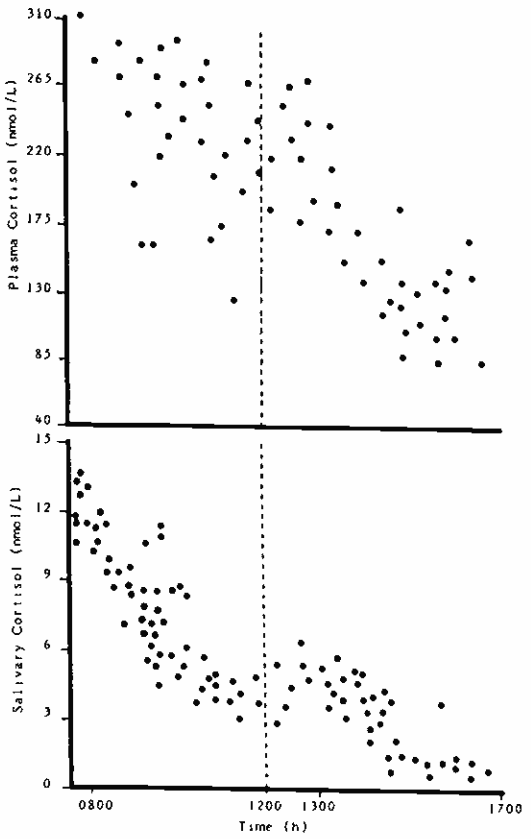


Table I - Diurnal variation in plasma and salivary cortisol concentrations in normal subjects

	Cortisol (nmol/L)		
Time (h)	Men (n=20)	Women (n=20)	Total (n=40)
0800-1000			
Plasma	225±67	213±59	221±64
Saliva	8.3±5.0	9.5±4.7	8.7±4.8
1500-1700			
Plasma	171±54	173±65	172±59[a]
Saliva	2.2±0.8	2.8±1.3	2.4±1.1[b]
Mean ratio AM/PM			
Plasma	1.32	1.23	1.28
Saliva	3.77	3.39	3.62

Values are mean ± 1SD
a $p<0.05$, b $p<0.001$

to the corresponding more consistent values from salivary measurements (Fig 1). This is further illustrated by the lower mean difference of morning and afternoon cortisol levels of 1.3 for plasma compared to a greater mean difference of 3.6 for salivary cortisol (Table I). There was a highly significant ($p<0.001$) diurnal variation of salivary cortisol concentrations whereas a p value of only 0.05 was obtained for total plasma levels (Table I). There was no significant difference between male and female cortisol concentrations.

The expected changes in total plasma cortisol levels after cortisone or dexamethasone treatment in normal subjects were also obtained from salivary measurements (Table II). Both total plasma and salivary cortisol levels were significantly ($p<0.001$) reduced by about 0.3 fold to 57±15 and 2.7±0.7 nmol/L respectively after dexamethasone treatment (Table II). In contrast, when normal subjects were given cortisone acetate, salivary cortisol levels were markedly elevated 3.5 fold to 26.3±8.9 nmol/L ($p<0.001$) while corresponding total plasma levels were only raised 2.3 fold to 475±124 nmol/L (Table II).

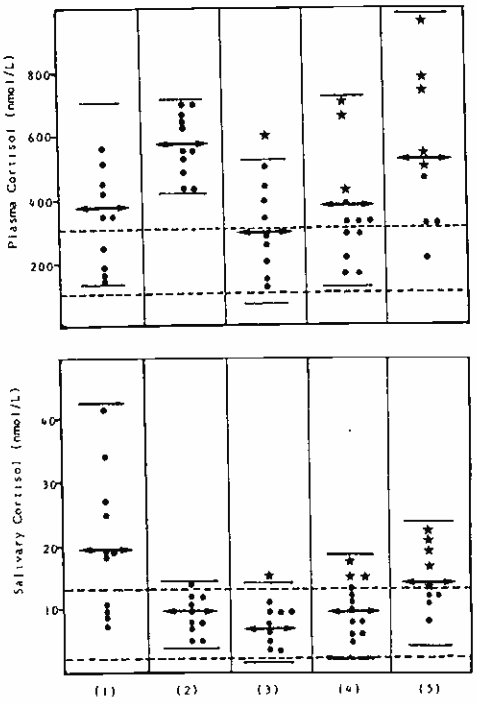
Fig 2 shows the comparison of simultaneous measurements of raised cortisol from morning samples of plasma and saliva of pregnant women and in patients treated for thyrotoxicosis, diabetes and hypertension as compared to the cortisone acetate group. Salivary cortisol levels in pregnancy samples (9.2±2.5 nmol/L) were within the normal reference range although the corresponding total plasma values were significantly ($p<0.01$)

Table II - Comparison of plasma and salivary cortisol concentrations in morning samples from subjects given cortisone acetate or dexamethasone

	Cortisol (nmol/L)		
	Normal (n=60)	Cortisone (n=11)	Dexamethasone (n=9)
Plasma	204±55	475±124[a]	57±15[a]
Saliva	7.5±2.6	26.3±8.9[b]	2.7±0.7[b]
Mean ratio/Normal			
Plasma		2.33	0.28
Saliva		3.51	0.36

Values are mean ±1SD
a $p<0.001$ versus normal plasma
b $p<0.001$ versus normal saliva

Fig 2 - Comparison of plasma total and salivary free cortisol concentrations for various categories of normal subjects and patients. (1) Normal treated with cortisone acetate (n=11); (2) Normal pregnant (n=11); (3) Thyroid (n=10); (4) Hypertension (n = 12); (5) Diabetes (n=9)



elevated to 565±72 nmol/L (Fig 2). From a total of 31 patients studied, only 9 (29%) had elevated salivary cortisol levels out of the 18 (58%) (5/10 thyroid, 7/12 hypertension and 6/9 diabetes) that showed elevated total plasma values (Fig 2 and Table III). The majority of patients that showed raised cortisol levels in both saliva and plasma were diabetic patients (5/9, 56%) followed by patients with hypertension (3/9, 33%) (Fig 2).

The overall relationship of salivary cortisol levels to that of time-matched total plasma values is shown in Fig 3. There was a good correlation (r ranging from 0.620 to 0.854 $p<0.01$ or $p<0.001$) in all individual normal or patient groups but no correlation ($r=0.180$, $p>0.5$) in pregnancy samples. After exclusion of data from pregnant and cortisone acetate groups, linear regression analysis of all the remaining data (n=91) gave a significant ($p<0.001$) coefficient correlation $r=0.805$ and $y=0.03x + 0.88$. As the normal upper limit for salivary cortisol of morning (0800-1300) samples (n=60) was established as 12.7 nmol/L, the corresponding value for total plasma cortisol was 394 nmol/L as derived from the linear regression equation.

DISCUSSION

Our in-house cortisol RIA which has been fully validated, is specific, sensitive and reproducible with inter-assay imprecision of less than 10%. As the assay is highly sensitive, only small volumes of samples (1.25 and 20 ul for plasma and saliva respectively) are required, thus making the assay suitable for measuring low levels of the free fraction of cortisol in saliva. The sensitivity of 1.5 nmol/L is comparable to that reported by Vining et al [3] who used 50 ul saliva. It is obvious that radioimmunoassays [5,7,8] are more sensitive and superior

Fig 3 - Correlation of salivary free cortisol levels to plasma total concentrations in time - matched samples (n = 91). Results from cortisone and pregnant groups were excluded in the final linear regression analysis which gave r = 0.805, y = 0.03x + 0.88, p<0.001

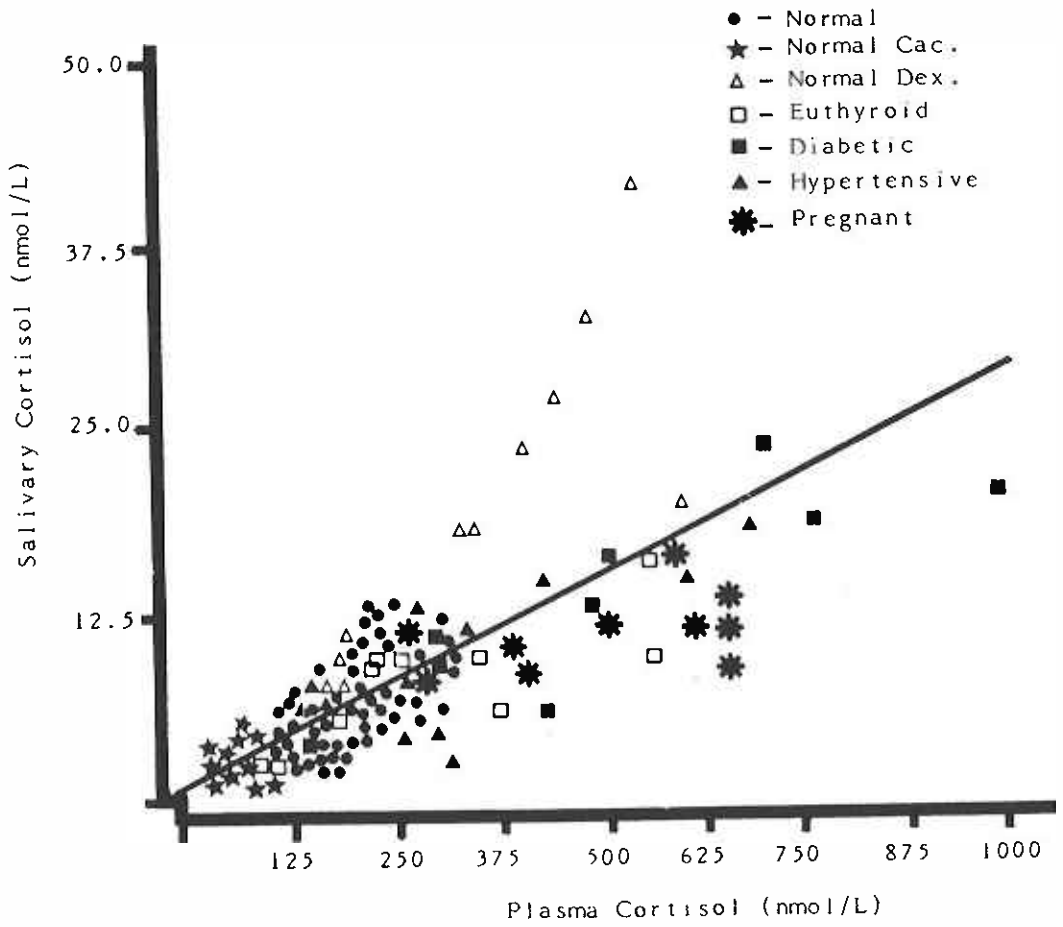


Table III - Comparison of plasma total and salivary free cortisol concentrations in morning samples from patients treated for thyrotoxicosis, diabetes and hypertension

			Cortisol (nmol/L)	
	No.	(%)	Plasma	Saliva
Normal Plasma Normal Saliva	13	(42%)	228±75	6.3±3.0
High Plasma Normal Saliva	9	(29%)	339±86[a]	6.9±2.6
High Plasma High Saliva	9	(29%)	663±169[a]	17.3±3.8[b]

Values are mean ±1SD
a $p<0.001$ versus normal plasma
b $p<0.001$ versus normal saliva

than the cortisol binding globulin (CBG) radiocompetition method used by Laudat et al[9] in which a large volume (2 mL) of saliva was required. The normal reference range for salivary cortisol in early morning samples 8.7±4.8 nmol/L (n=40) determined by this study is in good agreement with earlier reports (mean reported values range from 9.4 to 13.7) that used similar radioimmunoassays by either in-house assays [5,8] or commercial kits [7,18].

Our studies of circadian variation in normal subjects showed that a more marked variation was obtained for salivary cortisol levels as compared to plasma. However, this finding was not observed by Vining et al[8] who suggested that saliva samples should be collected by patients at times closer to the peak and nadir of the circadian rhythm at 0700 and 2000 rather than at the office hours of 0900 and 1700 since trained staff for venepuncture is not required. The loss of circadian variation in proven hypercortisolism in Cushing's syndrome has been demonstrated with saliva samples[2,9].

Our results of the dexamethasone suppression test in normal subjects are in agreement with earlier reports[2,8,9,18]. The clinical usefulness of salivary cortisol measurements in dexamethasone suppression tests for patients with Cushing's syndrome[2,9] and in ACTH stimulation tests[2,8,9] to test adrenal reserve have also been proven. In contrast to plasma total levels, a greater increase was obtained for the free fraction as detected by salivary measurements after adrenal stimulation by ACTH[6,8,9]. The greater increase in plasma free cortisol concentrations is probably due to saturation of CBG[8] which was also observed in our cortisone acetate group. On the other hand, this report together with previous studies[5,8] have shown the dissociation between elevated plasma total cortisol and normal salivary cortisol levels due to increases in CBG levels and/or binding capacity in pregnancy or oral oestrogen contraceptive treatment.

There was a good linear correlation between salivary and plasma total cortisol concentrations in normal subjects and patients up to 1000 nmol/L in plasma. This indicates that CBG binding capacity was concomitantly increased in accordance with the hypercortisol states of the patients in contrast to the marked increase in salivary cortisol when hypercortisolism was artificially and transiently produced in the cortisone acetate group. Although up to 58% of the patients had elevated plasma total cortisol concentrations, hypercortisolism as defined by raised salivary levels was confirmed in only half of the group. Levels of plasma total cortisol of between 300-500 nmol/L were found to have normal saliva values, while results from the regression equation show that high saliva levels were obtained only when plasma levels exceeded 400 nmol/L. These findings are in agreement with the reported estimate of 400-500 nmol/L saturation dose for CBG binding[8]. We therefore conclude that salivary cortisol measurement is a highly advantageous technique, being simple, stress-free and non-invasive and a better index of adrenal status than plasma measurement. Further investigations are required to ascertain whether raised free cortisol levels affect the clinical outcome and/or are due to poor control of diabetic and hypertensive patients.

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