

## Salivary cortisol in the assessment of adrenocortical function in patients with pulmonary tuberculosis

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Adrenocortical function was assessed on the basis of changes in salivary cortisol in patients with pulmonary tuberculosis and the findings compared with those in healthy subjects. A method of direct radioimmunoassay of salivary cortisol was standardized and the sensitivity was 0.8 nmol/l. Cortisol levels in saliva were significantly higher in the patients than in the healthy subjects ( $P < 0.001$ ). The diurnal rhythm of cortisol secretion was disturbed in the patients with a significant increase in salivary cortisol beyond 1800 h. While dexamethasone caused an appreciable suppression (87%), stimulation with ACTH (tetracosactrin) resulted in a marked increase in salivary cortisol, the increase being significantly higher in the healthy subjects than in the patients ( $P < 0.001$ ). Attempts to classify subjects as positive or negative responders to tetracosactrin based on increases in salivary cortisol in relation to 'plasma cortisol changes were however not successful, as the agreement between the two methods ranged from 73 to 80 per cent with various criteria used.

Salivary cortisol is believed to be a better measure of assessing adrenocortical function than plasma cortisol as it reflects the biologically active 'free' fraction (more than 90% of circulating cortisol is bound to plasma proteins, particularly the glycoprotein; transcortin)<sup>1-6</sup>. Most radioimmunoassay (RIA) methods described for the determination of salivary cortisol concentrations employ 'in house' reagents (antisera and labelled antigens). Al-Ansari *et al*<sup>7</sup> have described a procedure adapting a commercial serum cortisol immunophase kit for assays in saliva. We followed with some modifications this procedure for use of the 'Amerlex' kits (Amersham, UK) supplied for cortisol assays in serum/plasma, for the determination of cortisol levels in saliva.

Tuberculosis has traditionally been believed to be one of the causes of adrenal insufficiency in man<sup>8</sup>. Data on the adrenocortical function based on plasma cortisol in these patients is limited<sup>9,11</sup>, while no data is available based on salivary cortisol. We recently undertook an investigation to study the adrenocortical function in patients with pulmonary tuberculosis employing the ACTH (tetracosactrin) stimulation and dexamethasone suppression tests, and a study of the diurnal variation of cortisol release, and the findings were compared with those in healthy subjects. Matched samples of blood and saliva were collected during this investigation; we have reported our findings based on plasma levels<sup>12</sup>, and we present in this report those based on

salivary cortisol together with the details of the procedure for assay of cortisol in saliva.

### Material & Methods

*Collection of saliva* : Salivary gland secretion was stimulated by masticating a piece of unflavoured, unsweetened chewing gum. The collected saliva was frozen (kept at  $-20^{\circ}\text{C}$  overnight); then thawed and centrifuged. The residue containing mucoproteins was discarded and the clear supernatant stored at  $-20^{\circ}\text{C}$  till assay.

*Assay of salivary cortisol* : The highest standard (1935 nmol/l) provided in the Amerlex kit was reconstituted in 500  $\mu\text{l}$  of distilled water and suitably diluted with 6 per cent human serum albumin (HSA) in the assay buffer (0.1 M phosphate buffer with 0.4 M sodium chloride containing sodium azide 1g/l and bovine serum albumin 1g/l – pH 7.4) to give standards ranging from 20 to 968 nmol/l. The radioimmunoassay control (C-15) was reconstituted in 1 ml of distilled water and was used as 'high' control (362 nmol/l), and this was diluted 1 in 5 with 6 per cent HSA in the assay buffer to give the 'low' control (72.4 nmol/l). To 10  $\mu\text{l}$  of the standards and controls (as above) were added 100  $\mu\text{l}$  of the assay buffer to provide standard cortisol concentrations of 2.0, 4.0, 8.0, 16.1, 32.2, 64.5 and 96.8 nmol/l and control concentrations of 36.2 and 7.2 nmol/l in a total volume of 110  $\mu\text{l}$ . Ten  $\mu\text{l}$  of 6 per cent HSA in the assay buffer was used in place of cortisol solutions to provide the Zero standard. The supernatants of the saliva samples were processed at the same time and to 100  $\mu\text{l}$  of these samples were added 10  $\mu\text{l}$  of 6 per cent HSA in the assay buffer. To each of the above, set up in duplicate, were added 50  $\mu\text{l}$  of each of the  $^{125}\text{I}$  tracer and the cortisol antibody suspension. The contents were thoroughly mixed on a vortex shaker and then incubated at  $37^{\circ}\text{C}$  for 3 h. They were then centrifuged at  $28^{\circ}\text{C}$  at  $1500 \times g$  for 30 min, the supernatants discarded and the precipitate counted for 3 min in a Kontron automatic gamma counter (Gammamatic II). A logit-log linear regression curve-fit programme was employed for the calculation of cortisol concentrations, the two coordinates being the log concentrations of the standards (abscissa) and the logit of the percentage bound (ordinate).

Plasma cortisol was determined according to the instructions of the manufacturers.

*Precision of the method* : To check the precision of the salivary method, 3 controls were employed; the radioimmunoassay control (C-15) supplied by Amersham was diluted 1 in 10 and 1 in 50, and Wellcontrol II of Wellcome Diagnostics was diluted 1 in 10 with 6 per cent HSA in the assay buffer. All three controls were set up in duplicate on 16 different occasions (days) and the intra- and inter-assay co-efficients of variation calculated.

*Recovery of added cortisol* : On each of 9 different occasions, cortisol concentrations of 12.5, 25.0 and 50.0 nmol/l were added to pooled saliva. The assays were set up in duplicate and the recoveries calculated.

*Stability of cortisol* : Cortisol concentrations in 40 samples of saliva collected during the tetracosactrin stimulation test were determined soon after collection and after storage at  $-20^{\circ}\text{C}$  for 4 months.

*Association between salivary cortisol and plasma cortisol* : The association was examined among 48 matched samples of plasma and saliva collected during the tetracosactrin stimulation test. The plasma 'free' cortisol fraction (unbound) was separated from the bound cortisol by ultrafiltration employing Centricon- microconcentrators of 2 ml capacity with a surface area of 0.92  $\text{cm}^2$  of the YM membrane (molecular weight cut-off: 10,000 daltons). Cortisol concentrations in plasma (total), in the plasma ultrafiltrate ('free' fraction) and in saliva were determined simultaneously, those in the ultrafiltrate being determined by the same procedure as that for salivary cortisol.

*Subjects* : These investigations were undertaken in sputum smear-positive patients with pulmonary tuberculosis aged 20-59 yr and healthy subjects aged 20-51 yr. For the tetracosactrin stimulation test, two groups of patients, one newly diagnosed and the other, who had had the disease or been on specific anti-tuberculosis treatment for more than 3 yr (chronic cases) were investigated. None of the subjects was diabetic or pregnant and none was on any form of steroid treatment at the time of investigation. Informed consent was obtained from the subjects and the study was cleared by the

Ethical Committee of the Centre.

*Diurnal variation of salivary cortisol* : Saliva was collected at 0800 h and then at 2-hourly intervals up to 2000 h from 7 newly diagnosed patients with pulmonary tuberculosis and 6 healthy subjects.

*Dexamethasone suppression test* : Saliva was collected between 0800 and 0830 h from 9 newly diagnosed patients of pulmonary tuberculosis. They were administered 1 mg dexamethasone orally at bed time (2200 h) the same day, and samples of saliva were collected again between 0800 and 0830 h the next morning.

*ACTH stimulation test* : The ACTH stimulation test was performed employing tetracosactrin, which has a steroidogenic activity similar to the natural corticotrophin. Saliva was collected between 0800 and 0830 h from 36 healthy subjects, 27 newly diagnosed patients and 12 patients with chronic pulmonary tuberculosis for the determination of basal cortisol levels and again at 1/2 h and 1 h after intramuscular administration of 0.25 mg (about 24 units) of tetracosactrin. These subjects were classified as positive or negative responders to tetracosactrin on the basis of plasma cortisol levels, the definition for a positive response being an increase in plasma cortisol of 200 nmol/l or more at 1/2 h and 1 h over the respective basal levels<sup>12</sup>. Attempts were made to evolve criteria to define a positive response to tetracosactrin on the basis of increases in salivary cortisol and then to validate these criteria in a fresh batch of 57 newly diagnosed sputum smear-positive patients of pulmonary tuberculosis.

Student's 't' test (paired and unpaired) was employed for testing the differences between the mean cortisol levels, the Chi-square test with Yates' correction for continuity to test the differences between the proportions, and the analysis of variance (ANOVA) to examine the precision of the method and also for testing the differences between the mean cortisol concentrations in the study of the diurnal variation of cortisol release. The sensitivity of the method was calculated as described by Osredkar *et al*<sup>13</sup>.

Tetracosactrin (synacthen) was gifted by Hindustan Ciba Geigy Limited and cortisol assay kits (Amerlex) were purchased from Amersham

International, England. Wellcontrol II was purchased from Wellcome Diagnostics, England.

## Results

A typical standard curve (logit-log) for the salivary cortisol estimation is depicted in Fig. 1. The sensitivity of the method, defined as the concentration of cortisol equivalent to 2 standard deviations below the binding of the Zero standard<sup>13</sup>, was 0.8 nmol/l. The binding of labelled cortisol to the antiserum in the absence of unlabelled cortisol (Zero standard) varied with the total counts, and ranged between 41.1 to 53.1 per cent during the course of these investigations. The precision of the assay, as assessed by the intra- and inter-assay co-efficients of variation together with the expected and observed mean values are presented in Table I. The variations observed are typical of a steroid RIA. In practice, the tubes were counted again or the assay repeated if the

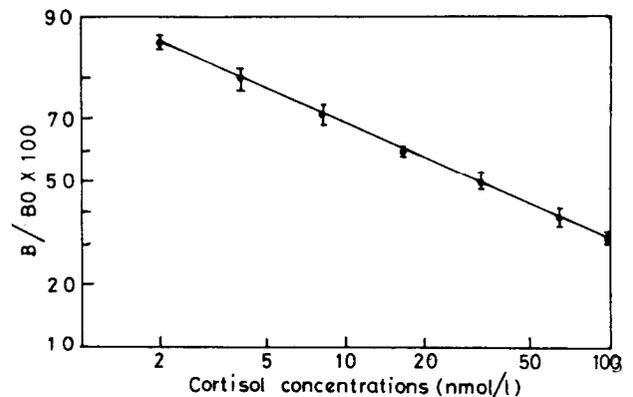


Fig. 1. Standard curve for assay of cortisol in saliva (vertical bars indicate the standard deviation).

**Table I.** Precision of the salivary cortisol assay

Control used	Expected mean value and range (nmol/l)	Observed mean value (nmol/l)	Co-efficient of variation (%)	
			Intra-assay	Inter-assay
Amersham C-15 (1 in 10)	36.2 (28.1-44.2)	43.3	8.5	7.8
Wellcontrol II (1 in 10)	27.3 (20.7-33.9)	24.8	10.5	13.3
Amersham C- I5 (1 in 50)	7.2 (5.6-8.8)	8.7	11.4	12.6

replicate variation in counts exceeded 10 per cent. The mean cortisol value of pooled saliva used for the recovery experiment was  $12.8 \pm 1.1$  nmol/l. The recoveries ranged from 83 to 124 per cent (mean, 96%) when 12.5 nmol/l of cortisol was added, from 73 to 105 per cent (mean, 87%) with 25 nmol/l, and 66 to 95 per cent (mean, 80%) following addition of 50 nmol/l. The mean cortisol concentrations of 40 saliva samples before and after storage for 4 months at  $-20^{\circ}\text{C}$  were  $34.7 \pm 21.9$  and  $34.3 \pm 24.1$  nmol/l, respectively ( $P > 0.2$ ). The association between salivary cortisol and plasma cortisol (total and 'free' fractions) was examined (Fig. 2). The correlation co-efficients between salivary cortisol and the total and 'free' fractions of plasma cortisol were 0.74 and 0.92, respectively ( $P < 0.001$ ).

**Diurnal variation of salivary cortisol :** The mean salivary concentrations of cortisol in the tuberculous patients were higher at all time-points than the corresponding mean values in healthy subjects (Fig. 3). In the healthy subjects, no significant change was seen in salivary cortisol till 1200 h; a decline was observed thereafter and the mean values at 1600, 1800 and 2000 h were significantly lower than that at 0800 h ( $P < 0.03$ ). In the patients, a slight increase was observed up to 1400 h followed by a decline and the mean values at 1600 and 1800 h were similar and lower than that at 0800 h ( $P = 0.05$ , for both). However, there was a rise thereafter and the mean value at 2000 h, was higher than those at 1600 or 1800 h ( $P = 0.01$ ).

**Dexamethasone suppression test :** There was an appreciable suppression (87%) of salivary cortisol following administration of dexamethasone to

patients with pulmonary tuberculosis, the mean salivary levels before and after dexamethasone being 16.3 and 2.1 nmol/l, respectively ( $P < 0.001$ ). The pre-and post-dexamethasone values correlated well ( $r = 0.79$ ;  $P = 0.01$ ).

**ACTH stimulation test :** The mean salivary concentrations of cortisol before (0 h) and at  $\frac{1}{2}$  h and 1 h after stimulation with tetracosactrin are presented in Table II. The mean basal saliva concentration (0 h) in the newly diagnosed patients was significantly higher than those in the healthy subjects ( $P < 0.001$ ) or the patients with chronic TB ( $P = 0.04$ ). At  $\frac{1}{2}$  h and 1 h, the mean concentration in the healthy subjects was similar to that in the newly diagnosed patients; the mean values in both these groups were, however, significantly higher than that in the chronic TB patients at both time-points ( $P < 0.01$ ). The association between base-line saliva levels and the increases at  $\frac{1}{2}$  h and 1 h (amalgamating the

**Table II.** Salivary concentrations of cortisol before and after stimulation with tetracosactrin

Group (n)	Basal value (0 h)	After tetracosactrin	
		$\frac{1}{2}$ h	1 h
Healthy subjects (36)	9.2 (3.1-21.3)	37.4 (15.8-57.7)	53.9 (26.3-83.7)
Newly diagnosed TB patients (27)	19.2 (4.4-61.1)	38.9 (16.2-80.2)	50.2 (29.6-104.5)
Chronic TB patients (12)	10.5 (6.2-20.4)	27.4 (18.6-40.6)	35.0 (11.6-59.7)

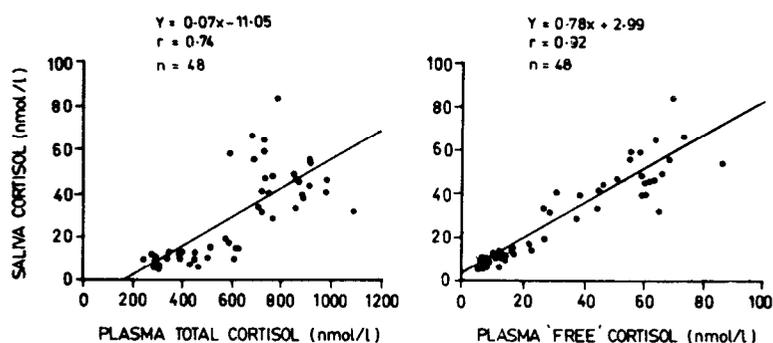
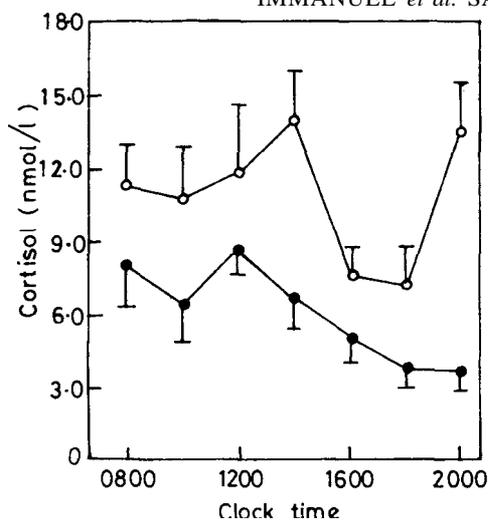


Fig.2. Association between salivary cortisol and plasma total or plasma 'free' cortisol.



**Fig.3.** Diurnal variation of salivary cortisol in healthy subjects (●—●) and patients with pulmonary tuberculosis (○—○). Vertical bars indicate the standard error of the mean.

findings in all 3 groups) was weak, the correlation co-efficients being  $-0.37$  at  $\frac{1}{2}$  h and  $-0.28$  at 1 h. The mean proportionate increases (%) over the basal level following stimulation with tetracosactrin in the 3 groups of subjects ranged from 163 to 394 per cent at  $\frac{1}{2}$  h and 254 to 617 per cent at 1 h. The magnitude of increase in salivary cortisol was significantly higher in the healthy subjects than in either group of tuberculous patients ( $P < 0.001$ ); the differences between the two groups of patients were, however, not significant ( $P > 0.2$ ).

*Classification as positive or negative responders to tetracosactrin* : Based on the increases in plasma cortisol at  $\frac{1}{2}$  h and 1 h, 34 (94%) of 36 healthy subjects, 13 (48%) of the 27 newly diagnosed patients and 5 (42%) of the 12 chronic TB patients had a positive response to tetracosactrin, the difference in the proportions between the healthy subjects and the 2 groups of patients being highly significant ( $P < 0.001$ )<sup>12</sup>. Attempts were made to derive suitable criteria to define a positive response on the basis of both the absolute increases and the proportionate increases in cortisol levels in saliva at  $\frac{1}{2}$  h, 1 h or both over the respective basal levels. Some of the criteria that provided the best estimates for sensitivity and specificity and agreement between the classifications based on the salivary and the plasma methods are listed in Table III. The sensitivity of the methods based on salivary concentrations

**Table III.** Estimates of sensitivity and specificity of salivary estimation, and agreement between the salivary and the plasma estimations in the classification of subjects as positive or negative responders to tetracosactrin

Criterion	Sensitivity (%)	Specificity (%)	Agreement (%)
<i>Based on absolute increase :</i>			
20 nmol/l or more at $\frac{1}{2}$ h	75	70	73
30 nmol/l or more at 1 h	79	83	80
20 nmol/l or more at $\frac{1}{2}$ h and 1 h	75	78	76
<i>Based on proportionate increase :</i>			
150% or more at $\frac{1}{2}$ h	79	61	73
300% or more at 1 h	73	87	77
150% or more at $\frac{1}{2}$ and 1 h	79	70	76

ranged from 73 to 79 per cent and the specificity from 61 to 87 per cent; the agreement in the classification between the two methods ranged from 73 to 80 per cent with the different criteria.

Attempts were made to validate these criteria in a fresh batch of 57 newly diagnosed patients of pulmonary tuberculosis, 28 (49%) of whom were classified as positive responders and the rest as negative responders to tetracosactrin on the basis of increases in plasma cortisol. The sensitivity of the salivary method ranged from 46 to 68 per cent and the specificity from 41 to 48 per cent when the 3 criteria based on absolute increases over the basal levels were employed. The estimates of sensitivity and specificity were higher with the criteria based on proportionate increases over the basal levels, the sensitivity ranging from 61 to 68 per cent and the specificity from 72 to 86 per cent for the 3 criteria listed.

## Discussion

Determination of the low concentrations of cortisol in saliva requires highly sensitive RIA procedures, and most investigators have employed 'in house' reagents (antisera and labelled antigens) for this purpose<sup>1-6</sup>. However, preparation of these reagents is restricted to specialized laboratories, and for routine use in clinical laboratories, it will be desirable to adapt commercially available kits designed for plasma/serum cortisol assays, for use in saliva. Al-Ansari *et al*<sup>7</sup> were the first to report

such an adaptation using the Corning Immuno-phase kit. We employed the 'Amerlex' kits and slightly modified the procedure of Al-Ansari and colleagues', the modifications aimed at improving the sensitivity of the method to provide a 'Zero' binding of approximately 50 per cent and to ensure that the counts of the Zero standard were at least 4 times higher than those with the highest standard employed. The minimum detectable limit was calculated to be 0.8 nmol/l as against 2.0 nmol/l reported by Al-Ansari *et al*<sup>7</sup>. With the volumes of the reagents provided with the 'Amerlex' kit, it is possible to undertake 400 determinations in saliva as against 100 in plasma/ serum. The precision of our assay is adequate, the variations observed being similar to those reported by Vining *et al*<sup>5</sup> and Al-Ansari *et al*<sup>7</sup>. The findings of the recovery experiments, however, suggest that it will be preferable to dilute the saliva sample 1:1 with the assay buffer if the cortisol concentrations exceed 50 nmol/l. Our findings also suggest that cortisol concentrations in saliva are stable for at least 4 months when stored at -20°C. According to information provided with the Amerlex kit, the antiserum supplied cross reacts strongly only with prednisolone (35%); the cross-reactivity with 24 other steroid hormones and their metabolites was negligible (< 2%). Since we used the same antiserum, we did not undertake any further studies on the specificity of the assay.

Stress induced by the disease is possibly responsible for the substantially higher salivary and plasma cortisol levels in newly diagnosed patients with pulmonary tuberculosis than in healthy subjects. The cortisol levels in chronic TB patients are significantly lower than those in the newly diagnosed patients, possibly due to the infiltration of the adrenals by the tubercle bacilli.

The tetracosactrin stimulation test is employed to examine the secretory potential of the adrenal cortex, and accurately reflects the integrated hypothalamic, pituitary and adrenal function<sup>14</sup>. Similar to our observation with plasma levels<sup>12</sup>, the rise in cortisol levels in saliva is much higher in the healthy subjects than in the tuberculous patients. A wide variety of definitions are available in the literature to define a normal (positive) response to tetracosactrin based on the

increases in plasma cortisol levels<sup>12</sup>. However, to the best of our knowledge, none is available to define a positive response on the basis of salivary cortisol. The increase in cortisol levels in saliva was disproportionately higher than that in plasma following stimulation with tetracosactrin. Thus, the mean increases in saliva in the 3 groups of subjects investigated ranged from 163 to 394 per cent at ½ h and 254 to 614 per cent at 1 h; the corresponding ranges in plasma were 49 to 115 and 64 to 148 per cent, respectively<sup>12</sup>. It is well known<sup>15</sup> that the binding sites of the cortisol-binding protein, transcortin, get saturated when plasma levels exceed 500 nmol/l. Thereafter, the increased plasma cortisol would be redistributed between the albumin-bound and 'free' fractions, with a disproportionate increase in the plasma 'free' moiety, which would be reflected in the salivary concentrations of the hormone. Despite the linear association between salivary cortisol and plasma free cortisol, we did not succeed in our attempts to derive criteria based on the increase in the former (either absolute levels or proportionate increases) for classification of subjects as positive or negative responders to tetracosactrin in relation to the classification based on plasma cortisol levels. The required levels of sensitivity and specificity (> 90% for both) were not attained and this was perhaps due to inter-individual variation in plasma transcortin concentrations. Peters *et al*<sup>16</sup> have observed that it is not possible to extrapolate normal ranges in plasma to provide equivalent concentrations in saliva in view of the inter-subject variation in the plasma binding capacity and degree of saturation of transcortin. However, in situations where the saturation capacity of transcortin is not likely to be exceeded, such as in the study of the diurnal rhythm of cortisol secretion and the dexamethasone suppression test, changes in the salivary levels can fairly reflect those observed in plasma.

In newly diagnosed patients of pulmonary tuberculosis, dexamethasone caused a significant suppression of salivary cortisol despite the high circulating levels, a finding similar to that observed in plasma cortisol levels<sup>12</sup>. This suggests a normal activity of the pituitary in these patients. The diurnal rhythm of cortisol secretion appears to be disturbed in patients with tuberculosis with

an evening rise in salivary cortisol and this phenomenon is similar to that reported earlier with plasma levels<sup>12</sup>. High levels of cortisol in saliva and a similar disturbed pattern were also observed before start of treatment in a fresh batch of 57 patients with pulmonary tuberculosis; the levels and the pattern returned to normal after 2 months of treatment with effective regimens of antituberculosis drugs (unpublished findings). The reasons for the evening rise in cortisol levels in these patients are not clear.

The findings reported here demonstrate that for most tests of adrenocortical function, with the exception of the tetracosactrin stimulation test, salivary cortisol is as good a measure as plasma cortisol and has the advantage of the collection procedure being stress-free and non-invasive. These findings and those reported earlier on plasma levels<sup>12</sup> also suggest that adrenocortical function is compromised in patients with pulmonary tuberculosis. It will be worthwhile to study the function of adrenal cortex in patients with extra-pulmonary tuberculosis, particularly tuberculous meningitis and brain tuberculoma.

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