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# Cortisol and Behavior: 1. Adaptation of a Radioimmunoassay Kit for Reliable and Inexpensive Salivary Cortisol Determination

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KIRSCHBAUM, C., C. J. STRASBURGER, W. JAMMERS AND D. H. HELLHAMMER. *Cortisol and behavior: 1. Adaptation of a radioimmunoassay kit for reliable and inexpensive salivary cortisol determination.* PHARMACOL BIOCHEM BEHAV **34**(4) 747–751, 1989.—We adapted a commercial serum cortisol radioimmunoassay (RIA) kit for use with saliva specimen. Using 50 μl sample volume, the lower sensitivity was found to be 0.02 μg/dl with intraassay variation coefficients between 5.4 and 8.9% at different concentrations. The 50% intercept was either 0.5 or 0.26 μg/dl (50 or 100 μl standard/sample volume). Fifty-four early morning samples from healthy adults showed absolute concentrations which are closely comparable to respective data from other laboratories. A comparison of 35 saliva samples which were each assayed with the adapted RIA as well as with three other commercial kits revealed high correlations between these assays ($r = .94$ to $r = .97$). Data on salivary cortisol responses to CRH stimulation and dexamethasone suppression in healthy subjects further the validity of the assay results. The most important contribution of this assay modification, however, is thought to be its impact on analysis costs: The protocol presented in this paper allows for reliable salivary cortisol measures with a reduction of costs for analytical material to 25% compared to serum determinations.

Cortisol Saliva Radioimmunoassay HPA axis

ASSESSMENT of cortisol has frequently been used as an indicator of hypothalamus-pituitary adrenal (HPA) activity. Especially under stressful stimulation, levels of this glucocorticoid can vary considerably with psychological variables being among the most potent stimuli to release this hormone from the adrenal cortex [for review see (20,23)]. Due to the fact that cortisol is a hydrophobic and rather small molecule, it is present in all bodily fluids and its concentration in saliva reflects the biologically active unbound plasma fraction of this steroid. Hence, samples can be obtained by the subjects themselves at any desired rate or duration, thus facilitating studies inside as well as outside the laboratory.

In contrast to total cortisol in plasma, salivary cortisol levels are not altered by estrogen containing medication (5,28). Therefore, women taking oral contraceptives as well as pregnant women (up to the third trimester) can be included in studies without doubts as to the comparability of hormone data. While the stress of venipuncture per se has been shown to increase cortisol levels in some subjects, there are no such effects in saliva sampling. Thus, cortisol in saliva appears to be advantageous for investigations of adrenal activity, having numerous advantages over blood sampling.

One essential requirement, however, is the availability of a sensitive assay which allows reliable cortisol determination in saliva at reasonable costs. Many workers have used 'in-house' reagents to perform radioimmunoassays (RIAs) of salivary cortisol (5, 7, 10, 13, 15, 18, 19, 21, 25, 29, 31), but these materials are usually inaccessible for other investigators. Several research groups, therefore, had to adapt commercial serum cortisol RIA kits for use with saliva specimen (1–4, 6, 9, 11, 14). But even with these modified kits, research in psychoendocrinology has still been hampered and restricted by high costs.

In 1982, Al-Ansari and co-workers published an adaptation of a commercial serum cortisol RIA kit for saliva determinations which was described as a simple and inexpensive method (1). However, since this assay kit has essentially been modified by the producer, a modification and evaluation of the new material for use with saliva samples is necessary. In this paper we would like to report on a simple adaptation of the new material which allows for inexpensive, but reliable salivary cortisol determinations. Compared to serum cortisol measure, we are now able to cut down costs for analytical material to 25%.

## METHOD

### *Saliva Samples*

Saliva was collected with the "Salivette" sampling device

TABLE 1

ASSAY SCHEMES FOR CORTISOL DETERMINATIONS WITH THE "MAGIC CORTISOL" RIA IN SERUM OR SALIVA

| Serum Assay | Saliva Assay |
|---|---|
| | dilute standards 1:10 in PBS |
| | ↓ |
| 10 µl standard/sample | 50 µl or 100 µl standard/sample |
| + | + |
| 500 µl antibody-solid phase solution | 100 µl antibody-solid phase solution |
| + | + |
| 100 µl $^{125}$J-tracer solution | 50 µl $^{125}$J-tracer solution |
| ↓ | ↓ |
| vortex | vortex |
| ↓ | ↓ |
| incubate 30 min at 37°C | incubate 3.5 hr at room temperature |
| ↓ | ↓ |
| 5 min magnetic separation | 5 min magnetic separation |
| | ↓ |
| | wash each tube with 1 ml dest. water |
| ↓ | ↓ |
| | 5 min magnetic separation |
| | ↓ |
| measure activity in γ-counter for 1 min | measure activity in γ-counter for 1 min |

(Sarstedt Inc., Rommelsdorf, F.R.G.) which mainly consists of a small cotton swab and two plastic tubes (12). The swab was left in the mouth for 1–5 minutes and subjects gently chewed on it to stimulate saliva flow. Recently, we could show that this sampling procedure per se has no influence on cortisol levels (16). After collection, the devices were stored at −20°C. Prior to assay, the devices were centrifuged for 2 minutes at 3000 rpm giving clear and watery samples.

*Assay Modification Protocol*

Table 1 gives the assay scheme for serum cortisol determination as opposed to the adapted scheme for salivary cortisol measures using the "Magic Cortisol" RIA (Ciba-Corning; Gießen, F.R.G.). The kit was modified as follows: Serum standards were diluted 1:10 with phosphate-buffered saline (100 mmol/l, pH 8.0) containing 0.1% sodium azid giving final standard solutions of 0, 0.1, 0.25, 0.6, 1.5, 3.5 and 7.5 µg/dl. To 50 µl standard or unknown sample, 100 µl antibody-solid phase solution (antibodies covalently bound to paramagnetic particles) as well as 50 µl tracer was added and the tubes were vortexed. All determinations were performed in duplicates. The incubation time was prolonged to 3.5 hours at room temperature. After incubation, the racks were placed on a separator unit containing 60 small magnets (Distribution by Ciba-Corning) for 3 minutes. Then, the liquid phase was decanted and 1 ml distilled water was added to each tube. After another separation step the liquid phase was again decanted and the tubes were subsequently counted for 1 minute in a 12-channel gamma-counter (Berthold, F.R.G.).

In another experiment, 100 µl standard or unknown sample were used keeping all other steps unaltered. Using this protocol, one assay kit for 100 serum samples allows for the determination of up to 500 saliva samples while only 15 ml tracer solution needs to be purchased separately.

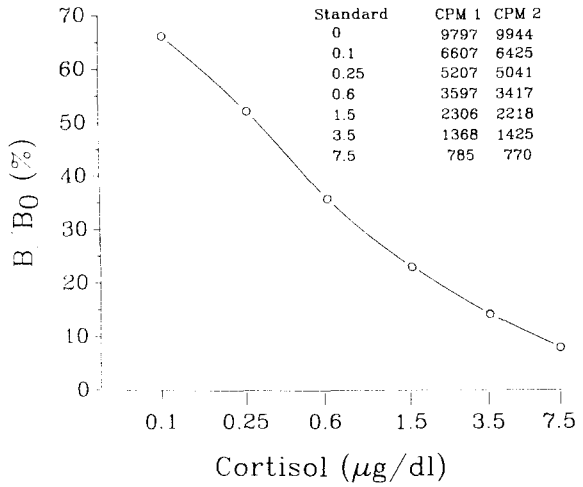


FIG. 1. Typical standard curve obtained with 100 µl of standard solution with absolute counts per minute for duplicate determinations (CPM 1 and CPM 2, respectively) for each calibrator.

*Comparison With Three Other RIA Kits*

In an attempt to estimate how closely results obtained with the adapted "Magic Cortisol" RIA are comparable with standardized test kits, 35 samples were additionally measured with three commercial RIAs ("Coat-a-Count," Diagnostic Products Inc., USA; "Corti-Cote," Becton-Dickinson, F.R.G.; "SPAC-Cortisol," Byk-Sangtec, F.R.G.). All of these tests employ the coated tube technique with antibodies being immobilized at the inner wall of each test tube. Two hundred µl ("Coat-a-Count") or 100 µl ("Corti-Cote" and "SPAC") standard and sample are followed by 1, 0.5 and 1 ml radioactive tracer solution, respectively. After incubating either overnight or 4 or 3 hours at room temperature and a subsequent washing step, the tubes were decanted and counted for 1 minute. Cortisol results from the adapted Corning assay and the three other RIA kits were then compared by linear regression analysis.

*Dynamic Tests of HPA Activity*

In order to evaluate the potential usefulness of the "Magic Cortisol" assay for the clinical endocrinologist we investigated salivary cortisol responses to HPA-axis challenge tests. Eleven healthy volunteers were injected IV with 100 µg h-CRH (Bissendorf, F.R.G.) in the afternoon. Saliva samples were obtained at −5, 15, 30, 45, and 60 minutes following peptide administration for monitoring changes in cortisol concentration. Furthermore, 6 volunteers took 1 mg dexamethasone at 11 p.m. and obtained a saliva samples at 8:00 hr the next morning. The same subjects collected another sample on a drug-free control morning to determine a normal early morning cortisol concentration.

## RESULTS

*Assay Characteristics*

The antiserum has a crossreactivity of 31.4% for prednisolone, 5.0% for 11-deoxycortisol and less than 1% for cortisone, dexamethasone, progesterone, and testosterone. The lower detection limit at the 95% confidence interval was found to be 0.02 µg/dl for

TABLE 2

INFLUENCE OF TUBE POSITION ON SALIVARY CORTISOL MEASURE

| Tube Position No. | Concentration (μg/dl) |
|---|---|
| 60 | 0.558 |
| 120 | 0.523 |
| 180 | 0.508 |
| 240 | 0.549 |
| 300 | 0.569 |
| 360 | 0.500 |
| 420 | 0.538 |
| 480 | 0.606 |

our adaptation of the "Magic Cortisol" assay. A typical standard curve is depicted in Fig. 1.

With possible 50% intercept at 0.5μg/dl (50 μg/dl standard/sample) and 0.26 μg/dl (100 μg/dl standard/sample) the assay sensitivity can be switched to the concentration range expected in the investigated samples. Twenty-fold determinations of five different saliva pools (0.17, 0.3, 0.54, 0.71 and 1.36 μg/dl) revealed intraassay coeffients of variance of 7.3, 8.2, 8.9, 8.9 and 5.4%, respectively. Between-assay variation was found to be 10.1% (0.54 μg/dl; n = 12).

We measured 54 morning (8 a.m.) saliva samples from healthy adults for cortisol to compare the absolute concentrations obtained with the "Magic Cortisol" kit with data from other laboratories. We found early morning values between 0.45 and 0.78 μg/dl which is in agreement with results previously reported (22). This observation suggests that the Corning assay is not significantly affected by crossreactivity or matrix effects.

In order to investigate whether the tube position has any systematic effect on assay results, we measured a control sample 8-fold during one assay at different time points. Table 2 shows the concentrations at various tube positions. With a mean value of 0.544 μg/dl and a coefficient of variation of 6.37%, we were unable to detect any systematic alteration in these eight samples depending on tube position. Thus, more than 250 samples can be run in one assay without any systematic alteration of steroid values.

*Comparison With Three Other Commercial RIA Kits*

Thirty-five saliva samples were each assayed with the adapted "Magic Cortisol," the "Corti-Cote," the "Count-a-Count," and the "SPAC-Cortisol" kit to estimate the concordance of cortisol concentrations obtained with the adapted Corning test when compared to different assays. Figure 2 illustrates the results of three linear regression analyses.

Our adapted Corning RIA showed similar results compared to the "Corti-Cote" and "Count-a-Coat." The multiple correlation coefficients were r = .94 ("Magic Cortisol" RIA vs. "Coat-a-Count") and r = .95 ("Magic Cortisol" RIA vs. "Corti-Cote"). The absolute concentrations were closely comparable between these tests, although the values measured with the Corning assay appeared to be slightly higher. In contrast, although we observed a high linear correlation coefficient of r = .97 between the "Magic Cortisol" and the "SPAC-Cortisol" results, concentrations were found to be more than 2-fold higher in samples when measured with the "SPAC" kit (y = 2.24x − 0.09 for "Magic Cortisol" vs. "SPAC"). The standard solutions of the "SPAC" kit were assayed with the "Magic Cortisol" kit and found to correspond to the concentrations stated on the vials. One possible explanation for the high absolute values given by the "SPAC" kit is that the antiserum recognizes other salivary components to a considerable amount.

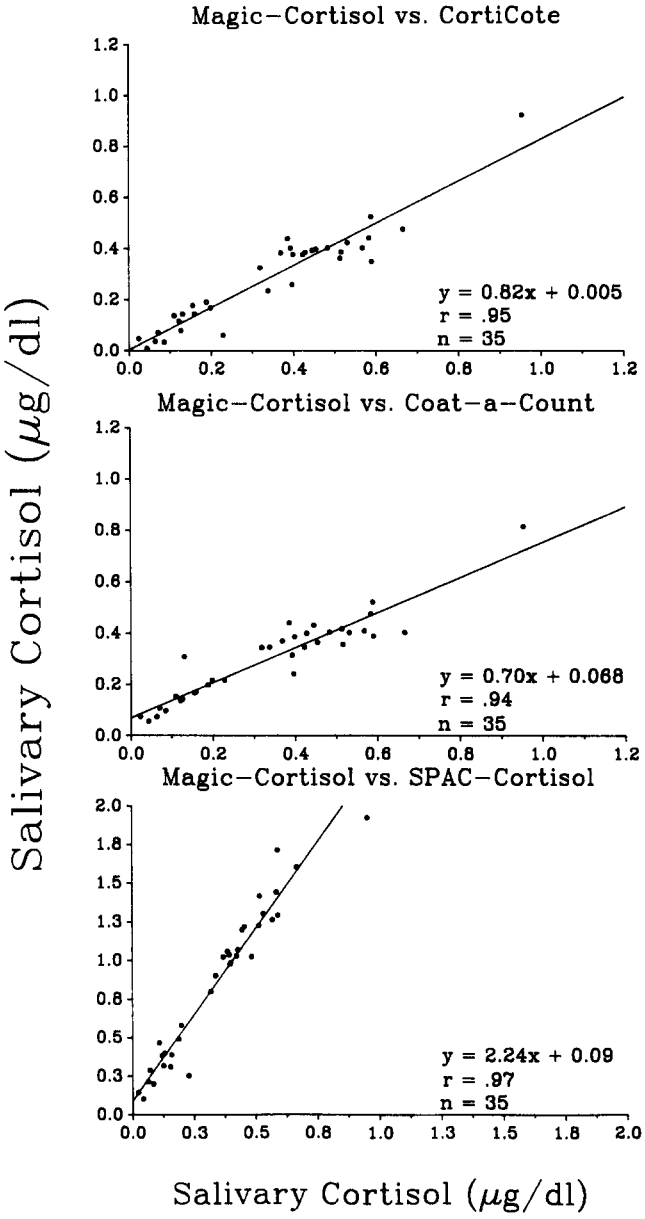


FIG. 2. Linear regression results comparing absolute concentrations of saliva samples (n = 35) assayed with the "Magic Cortisol" with results from the same samples assayed additionally with three other commercial radioimmunoassay (RIA) kits. Regression equations and multiple correlation coefficients (r) are shown in the respective plot.

*CRH Stimulation and Dexamethasone Suppression*

Figure 3 shows the mean salivary cortisol response to 100 μg h-CRH in 11 healthy subjects. From a baseline value of 0.12 μg/dl (±0.02, SEM) cortisol levels steadily rose to a peak concentration of 0.72 μg/dl (±0.10, SEM) at 45 minutes postinjection. The

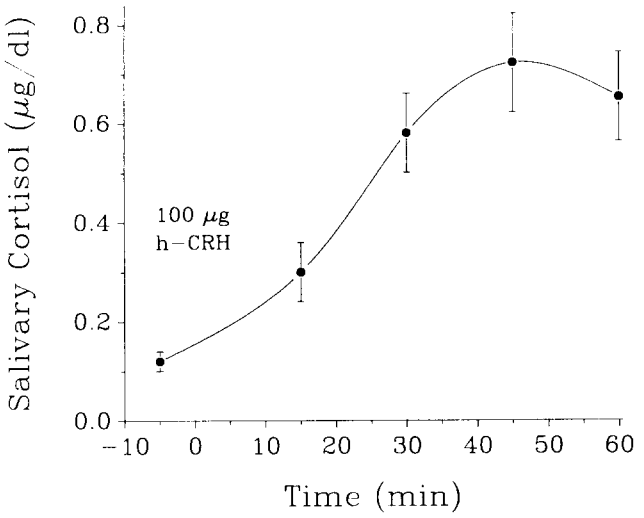


FIG. 3. Salivary cortisol responses to injection of 100 μg h-CRH in 11 healthy subjects.

peptide injection induced a significant increase in salivary cortisol levels in each volunteer, however, we observed large interindividual differences in adrenal reactivity. The absolute increases in cortisol ranged from 0.26 μg/dl to 1.10 μg/dl representing elevations of 223% and 2223% compared to baselines, respectively. Responses to dexamethasone suppression are shown in Fig. 4. While normal salivary cortisol concentrations ranged from 0.40 to 0.99 μg/dl in our volunteers at 8 a.m., all six subjects had cortisol values below 0.03 μg/dl after a standard over night dexamethasone suppression test. These results further support the validity of salivary cortisol measures employing our adaptation of the Corning "Magic Cor" RIA in clinical endocrinology.

## DISCUSSION

Assessment of salivary cortisol has recently come to be considered the method of choice for measuring cortisol in man

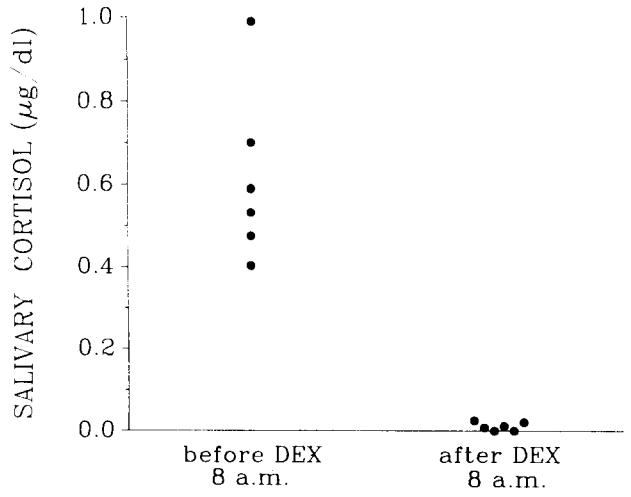


FIG. 4. Suppression of salivary cortisol after overnight dexamethasone suppression in 6 healthy adults. Each open circle represents a single cortisol value before and after oral intake of 1 mg dexamethasone.

(28), because it circumvents several problems associated with plasma or serum determinations. Easy and stress-free sampling regimen (12), its independence from corticosteroid binding globulin (8), and the well documented high correlations with plasma-free cortisol (27,28) are predominant features of salivary cortisol determinations. In the past, the determination of cortisol with commercial reagents was relatively expensive. Often the budget for sample analyses restricted extensive studies of the interaction between cortisol and behavior. Only few groups could afford sampling at short intervals in large populations, because they had 'in-house' reagents for cortisol analysis. This situation forced other investigators to adapt commercial RIA kits. Al-Ansari *et al.* (1) reported on an adaptation of a serum cortisol RIA which allowed 800 determinations with a 100-tubes kit. Although there is no information given about the absolute counts obtained with this protocol, one can easily conclude from the amount of tracer added to each tube that the total activity was less than 5000 cpm. In concert with a rather low dynamic range ($B_0/B_{7.5}$), this should have had an impact on assay reliability. Recently, the producer of this serum kit has essentially modified the assay introducing a new solid phase which allows for easy and quick separation of solid and liquid phase without a centrifugation step. We adapted the new assay for use in saliva and evaluated its performance.

With increased sample volume, smaller amounts of both antibody solution and tracer, and a single wash step, the Corning "Magic Cortisol" RIA was found to be a sensitive, reliable, and inexpensive method for cortisol measures in saliva. With a lower detection limit of 0.02 μg/dl and intraassay coefficients of variance between 5.4 and 8.9%, it is comparable to other sensitive in-house assays as well as commercially available RIAs (1, 3, 31). The absolute values obtained with the adapted "Magic Cortisol" RIA in different populations correspond to those reported by other groups (17). Since the incubation time can be a critical variable in immunometric assays, the possible influence of tube position on absolute values obtained has to be carefully ruled out. With a prolonged incubation of 3.5 hours and a quick separation step, we were unable to detect any systematic variation of cortisol concentrations throughout the course of one assay. More than 250 samples can be run in one assay without any significant shift of cortisol levels from the first to the last sample, which, in turn, reduces expenditure of labor and analytical error.

Being supplied with additional tracer, we are able to measure 500 tubes with a 100-tubes kit, thus cutting down the cost for analytical material to 25% as compared to regular kit purchase. Besides spending a considerable amount of money for each blood drawing (sterile disposable material, medical personnel, etc.) the serum cortisol analysis is much more expensive than the determination in saliva. Given a regular price of approximately 4 US$ for each serum analysis, salivary cortisol can now be measured for less than 1 US$ with the protocol outlined here. Investigators are now able to take samples at shorter intervals from more subjects, thus increasing the informative value and the accuracy of their studies.

Seth (24) pointed out that the absolute values obtained with different assay systems are found to vary considerably. Thus, comparisons of absolute hormone levels obtained with different RIA techniques are difficult or even impossible to interpret. In the face of this problem we measured 35 saliva samples with three different RIAs and found high correlations among them. While the adapted Corning assay, the "Corti-Cote" assay (Becton-Dickinson) and the "Coat-a-Count" RIA (Diagnostic Products) produced closely comparable values, the concentrations obtained with "SPAC-Cortisol" kit (Byk-Sangtec) were more than 2-fold higher. This finding is in line with a report of Tarui and Nakamura (26) who used the "SPAC" kit and found resting cortisol levels of 0.73 to 0.86 μg/dl in early afternoon saliva samples. Comparing these results with data from other laboratories (22), it is apparent

that the "SPAC Cortisol" kit may accurately reflect proportional alterations of salivary cortisol levels, but overestimates the absolute steroid concentration. Therefore, special care in interpreting data obtained with this kit in clinical investigations is called for. Our results on the cortisol response to h-CRH stimulation extended those Kahn and co-workers (14). Measuring salivary cortisol levels at 15-minute intervals following peptide injection, we monitored the peak hormone concentration at 45 minutes postinjection. Since Kahn *et al.* only took samples at 30 and 60 minutes they might have missed the peak cortisol levels in their subjects. In concert with the data on the dexamethasone suppression test, these results further support the usefulness of salivary cortisol measures in a clinical setting.

In conclusion, it can be stated that saliva as an alternative specimen for cortisol determination has proven a useful tool in studies of HPA activity. Employing our adaptation of the Corning "Magic Cortisol" RIA kit, one can now easily monitor salivary cortisol levels at low costs. This may help many investigators interested, e.g., in the dynamics of cortisol in response to stressful stimulations to expand their research activities on the psychoendocrinology of the hypothalamus-pituitary-adrenal axis.

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