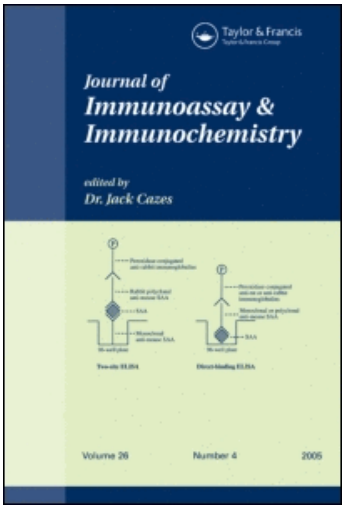

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Establishment and Application of Enzyme Immunoassay for Saliva Cortisol in Taiwanese Context

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Abstract: Saliva steroid assay is an upcoming area of research, with much potential for growth and progress. Expensive, varying results with commercial kits and the disadvantages of radioimmunoassay have forced researchers to develop their own system of enzyme immunoassay (EIA). A modification from our established EIA system was used to develop a saliva cortisol (F) assay system. The system sensitivity ($>90\,pg/mL$) was checked by various experiments, including comparison of data with a commercial kit obtained from Salimetrics®. The assay system was employed to investigate the saliva F level in a young Taiwanese population, and compared with the total and free serum levels of F.

Keywords: Saliva, Steroids, Cortisol, EIA, Taiwanese

INTRODUCTION

Saliva, as a biological fluid for alternative to measurement of steroid hormones in plasma, has been gaining popularity.[1,2] Salivary steroids assay has many advantages over plasma as a biological sample.[3] Frequent, easy collection by noninvasive, stress-free techniques makes it highly acceptable. Moreover,

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saliva collection for F assay rules out the false increase in needle-fearing patients, thus providing an ideal technique, especially for clinical assay of F hormone profile. Saliva providers find little difficulty in salivating directly into disposable tubes, providing adequate volumes for determining steroid profile, which can be done in a short time. The other advantage is the ease of storage, as saliva can be stored at -20°C for 6–9 months, at 4°C for 7 days, or at room temperature for 48 hours without any significant change in steroid amount.[4] The proteins and/or mucopolysaccharides can be sedimented by centrifuging the already frozen saliva sample. The risk of handling a saliva sample can be greatly reduced by sterilizing the sample at 60°C and more for up to thirty minutes, which does not seem to influence the steroid status in the sample.[5,6] Much of the earlier work in salivary steroids assay has been accomplished employing radioimmunoassay (RIA) techniques.[7,8] However, enzyme immunoassay (EIA) steroid assessment is now more popular, as it is safer, faster, and more reliable than RIA.[9] Cortisol (F) steroid hormone determination has been at the forefront of the ongoing research on saliva steroids.[10,11] In spite of the relatively lower amount of F in the saliva compared to the serum, radioimmunoassay[12,13] or enzyme immunoassay (EIA)[14,15] has successfully been used to assay it. However, commercial kits being expensive for routine use also varies in the assay results in different laboratory conditions. Hence, to provide a cheap source of F EIA kits, we have established a first of its kind of saliva cortisol assay system in Taiwan. Taiwanese populace, comprising a majority of Chinese ethnicity, have not been intensively studied for their endocrine profiles in saliva, especially in the field of steroid hormones. However, some work was reported recently on the onset of adrenarche in Taiwanese children,[16] and adrenal insufficiency in emergency department patients.[17] There is no established range of saliva, serum total and free F levels in the Taiwanese population, though some work on F secretion in the elderly, as influenced by gender, age, and disease condition in ethnic Chinese community, is reported.[18] This work is an announcement of our successful establishment of a saliva F EIA system, and its application to establish preliminary saliva, serum F levels in the Taiwanese populace.

EXPERIMENTAL

Antibody Production

The process for production of F polyclonal antibody was modified from that mentioned in our earlier work.[19] Rabbits were immunized with a mixture of Cortisol-11-α-ol-dione hemisuccinate: BSA and Freund adjuvants were administered once a week for a month, and then, once every month. The first immunization was with complete adjuvant, while later, incomplete adjuvant was used. The antibody titer was checked routinely after a month, and when an

appropriate titer level was detected, the animals were humanely slaughtered to obtain the antibody, which was diluted and stored for later use.

Enzyme Conjugate Preparation

Horseradish peroxidase (HRP, type VI, Sigma, P-8375) was coupled with Cortisol-11-α-ol-dione hemisuccinate, using a modification of the mixed anhydride method clearly described in our earlier work.[19] In brief, 1 mg of Cortisol-11-α-ol-dione hemisuccinate was dissolved in 250 μL of 1,4-dioxan. Then, ten μL of tri-n-butylamine and 5 μL of isobutyl chlorocarbonate were mixed into the solution. After half an hour of constant stirring in an ice bath, 1 mL of HRP (2,500 units/mL distilled water) was added at a controlled temperature of 8–10°C and pH 8.0. The reaction mixture was further stirred for four hours before subjecting it to dialysis, three times, with 0.05 M phosphate buffer of pH 7.0. This dialysate was then applied to a 1.6 × 100 cm Sephadex (Pharmacia) G-100 column and eluted with 100 mL of 0.05 M phosphate buffer (pH 7.0). In a cold room, the slow process of elution was monitored and Various fractions of the eluate were collected and subjected to measurement of enzymatic and immunologic activities. The fraction with the higher activity was chosen for use and, after diluting ten times, was stored in a small Ependorf at −20°C until used. This conjugated HRP was labeled as F-HRP, as mentioned later elsewhere in the text.

Enzyme Immunoassay Procedure

The EIA plates were prepared by coating the 96-well plates (Coster). Briefly, 200 μL of coating buffer (prepared in one liter of double distilled water (DDW)) containing 2.93 g of $NaHCO_3$ (Merck Art.6329), 1.59 g of Na_2CO_3 (Osaka TNO 461), Thimerosal 0.1 g (Sigma, T-5125) at a final pH of 9.6, containing polyclonal cortisol antibody at a dilution of 40,000x, was provided per well to the EIA plate. The antibody coated plates were stored overnight at 4°C following which they were washed with washing buffer (containing 10.86 g of $Na_2HPO_4 \cdot 2H_2O$, 5.38 gm of $NaH_2PO_4 \cdot H_2O$, 1 g of Thimerosal, and 10 mL of Tween-20 in ten liters of DDW at pH 7.0) before blocking with a blocking buffer. The blocking buffer (containing 5.0 g of gelatin, which was placed in 500 mL of DDW, subjected to continuous stirring and heating to 50°C for 15 minutes, after which the volume was raised to 2 liters by addition of DDW and added with 17.5 g of NaCl, 12.1 g of Tris-base (Sigma T-1503), 3.6 g of EDTA (Sigma), 1.0 mL of Tween-20 and the final pH of the buffer was maintained at 8.0); EIA plates were then sealed with a plastic plate sealer and stored at 4°C until further used.

At the time of assay, the saliva samples were taken from the refrigerator, thawed, and centrifuged. They, as well as the standards and quality control

samples, were then diluted with assay buffer in a recycled EIA plate. The diluted samples, standards, and QCs were then transferred to the cortisol antibody coated EIA plate in duplicate, 50 μL in each well. Ten times diluted F-HRP from original stock was further diluted to 1,500 times with assay buffer and then added to the plate and incubated in a stirring condition at room temperature for an hour. The plates were then washed and 200 μL per well of phosphate buffer 6 (PB-6), containing 10 mg OD and 2.5 μl 3% hydrogen peroxide (H_2O_2) were added per plate. The plate was incubated for half an hour and, depending upon the color changes in the standard wells, the reaction was stopped with 50 μL of 8 N sulfuric acid (H_2SO_4). The plate was then read in an ELISA plate reader (Dynatech) at 490 nm and corrected by 630 nm.

The standards were prepared from cortisone (Sigma, Lot 13H0525). The standards were diluted with assay buffer to attain a final dilution of 156 pg/mL, 312 pg/mL, 625 pg/mL, 1.25 ng/mL, 2.5 ng/mL and 5 ng/mL from standard 2–7. The first well was left as a blank (air), while the second well contained ther same amount of assay buffer as the standards.

The quality control (QC) was obtained from the pooled saliva, which was charcoal extracted and adjusted, with external steroid addition, to 200 pg/mL as QC Low, and 2.5 ng/mL as QC High.

The cross reaction of the antibody due to the presence of other steroids was monitored using a slight modification of our established method.[20]

The sensitivity of the system was calculated by assay of 0, 156, 312, 625, 1,250, 2,500, and 5,000 pg/mL of F standards in five replicates; the observed OD value was statistically evaluated by a *t*-test, 95% confidence limit, to calculate the binding percentages; they were expressed as log values, and calculated as the mean $\pm$ SD. The sensitivity of this system was calculated from the average binding percentage of the blank minus 2 $\times$ SD, and then the concentration was obtained by the equation of the standard curve.

The precision of the system was measured by using three saliva samples collected in the morning and evening. The samples were adjusted to three different levels in the range of 0.27 ng/mL (low), 1.1 ng/mL (medium) and 5.98 ng/mL (high). The intra assay was determined by the simultaneous assay of five replicates of the added saliva samples, in the same and different plate tests; the coefficient of variation was calculated.

The recovery rate of the cortisol level for the low (0.27 ng/mL) and high (1.52 ng/mL) saliva was calculated by adjusting with added exogenous 0.31 ng/mL and 2.5 ng/mL cortisol, respectively. Five replicates for each concentration were assayed and the observed amount was compared with the expected amount and the recovery percentage calculated.

The linearity of dilution was calculated to ascertain the effect of interfering factors in the saliva and serum and to determine the suitable dilution factor for each.

The ratio of serum total, serum free, and salivary cortisol were also assayed. The serum and saliva samples of the same individual were assayed.

The serum free cortisol was obtained by precipitating the serum with an equal volume of saturated ammonium sulfate. The Eppendorf containing serum and ammonium sulfate was centrifuged at 3,000 *g*, 4x, and the clear fluid at the top was used for assay. The saliva sample was thawed, centrifuged at 3,000 rpm, and diluted two to three times prior to assay. In order to investigate the sensitivity and reliability of our system, we compared it with a commercial kit obtained from Salimetrics®.

Sample Collection, Preparation, and Storage

Twenty-five males and twenty-six females of an average age of 21 years (20–23) volunteered to provide the samples. After instructing them on the method of saliva collection, they were encouraged to drool the saliva. Saliva ranging in volume from one to three mL was expectorated into an Eppendorf guided through a plastic pipette tip in about 5–10 minutes. Saliva specimens were stored at -20°C until assayed. At the time of assay, the saliva specimens were thawed and centrifuged at 3,000 rpm for 15 minutes. The saliva was collected in the morning starting from 8–9 AM and in the evening at 3–4 PM; blood collection immediately followed the completion of saliva collection in a tube devoid of any anticoagulant. The coagulated blood was centrifuged at 3,000 rpm for 15 minutes and the serum was transferred into a new Eppendorf and stored at -20°C until further assayed.

Chemicals and Reagents

Commercially procured chemicals and reagents were used in these experiments. Cortisol-11-α-ol-dione hemisuccinate was purchased from Steraloids, Freund's complete adjuvant and Freund's incomplete adjuvant were procured from Sigma Q3900), 1,4-dioxane, tri-n-butylamine, isobutyl chloroformate, HRP (horseradish peroxidase), 0.1 N NaOH, 0.05 M PB 7.0 (phosphate buffer, pH 7.0), and other lab grade chemicals were procured commercially.

RESULTS

The established EIA system was checked for its cross-reactivity with other steroids; it was found that 17α-OH-progesterone, progesterone, corticosterone, and testosterone had notable cross-reactions, while they were negligible for other steroids, as shown in Fig. 1. Figure 2 shows the standard curve of the established system. Table 1 shows the recovery rate of the established EIA system, while Table 2 shows the inter- and intra-coefficient of variation. The presence of interfering factors in saliva influencing the assay system, and the effect of linearity of dilution are shown in Fig. 3. Figure 4

Steroid	Cross-reactivity %
Cortisol	100
Corticosterone	17.8
Cholesterol	< 0.01
Estrone	< 0.01
Estradiol	<0.01
Estriol	< 0.01
Pregnenolone	0.02
Progesterone	21.3
17α-OH Progesterone	66.2
Testosterone	4.9

Figure 1. Cross-reactivity percentage of various steroids with our used cortisol antiserum (No. 6762) determined by CR 50%. The test was performed by RIA, immunoantigen: 4-pregnen-11β, 17α, 21-triol-3, 20-dione 21-HS: BSA.

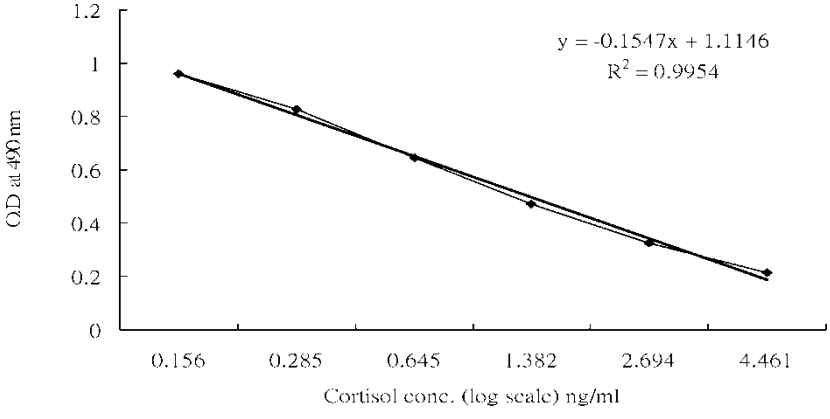

Figure 2. Standard curve of cortisol in relation to the optical density monitored by EIA plate reader in the range from 156 pg/mL to 5 ng/mL.

Table 1. The reliability of the system tested with the recovery percentage

Cortisol added recovery (ng/mL)	Sample	Cortisol endogenous concentration (ng/mL)	(ng/mL)	Expected (ng/mL)	Observed (%)
0.31	Low	0.26	0.57	0.44	75.8
	High	1.52	1.83	1.53	83.3
2.50	Low	0.26	2.76	2.78	100.7
	High	1.52	4.02	3.82	95.0

Table 2. The intra and inter-assay coefficient of variation

		Intra-assay			Inter-assay	
Sample	n	Mean ± SD ng/mL	CV (%)	n	Mean ± SD ng/mL	CV (%)
Low	5	0.27 ± 0.01	3.7	5	0.27 ± 0.05	17.8
Medium	5	1.10 ± 0.05	4.6	5	1.10 ± 0.20	18.7
High	5	5.98 ± 0.10	1.7	5	5.98 ± 1.06	18.0

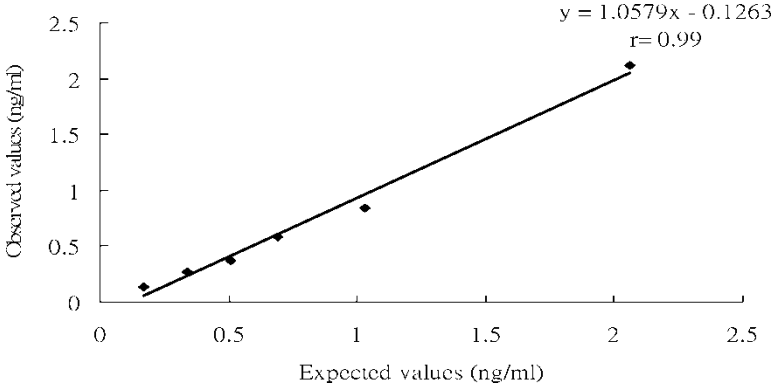

Figure 3. To investigate the presence of assay interfering factors in saliva, two samples of saliva were serially diluted with assay buffer and their F level assayed.

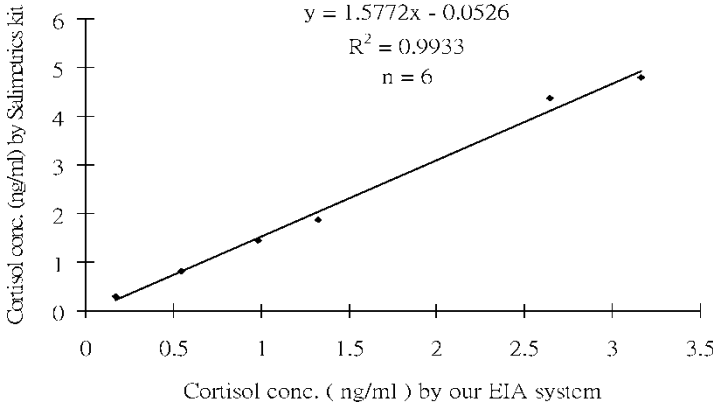

Figure 4. Comparative study of our established system with that of a commercial kit obtained from Salimetrics®.

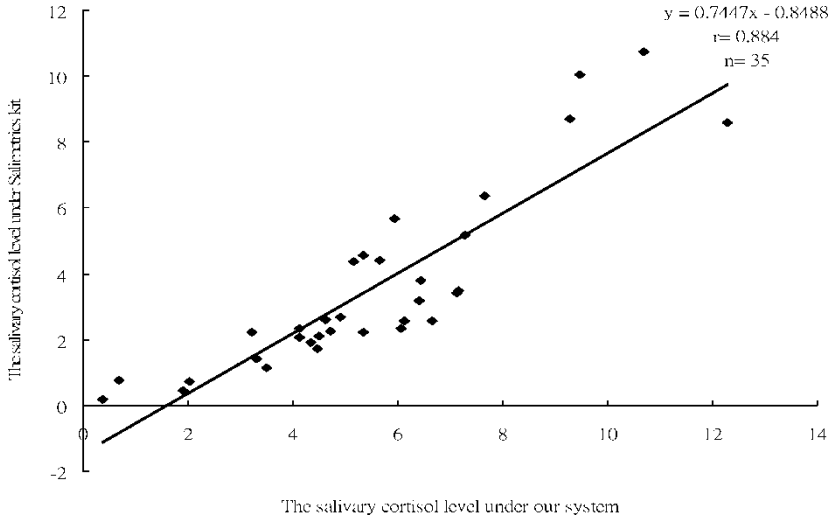

Figure 5. Correlative level of saliva F as monitored by the commercial kit obtained from Salimetrics® and our established system.

compares our established system with that of the commercial kit obtained from Salimetrics® for known saliva levels and Figure 5 shows it for unknown saliva F levels, respectively. Table 3, shows the F level in the saliva, as well as total and free F levels in serum, in young male and female Taiwanese volunteers, both in the morning and evening samples.

DISCUSSION

Stress related disorder is a severe challenge for the medical community, with various factors contributing to it.[21] Today's hectic life-style and lack of trend and facility to detect stress in its early stages are precipitating factors for many stress related disorders. Detection of stress can be achieved by monitoring the

Table 3. The level of saliva, serum total and free F level in young Taiwanese, assayed using our established system

Sex	Time	Serum F (ng/mL)		Saliva F (ng/mL)
		Total	Free	
Male	A.M.	209.06 ± 21.95	11.08 ± 2.93	9.77 ± 4.05
	P.M.	149.32 ± 34.44	5.56 ± 2.61	4.20 ± 2.85
Female	A.M.	186.34 ± 38.96	12.18 ± 5.74	9.95 ± 3.56
	P.M.	78.94 ± 39.54	6.32 ± 3.84	4.55 ± 2.61

body level of stress hormone F produced by the adrenal gland in response to stress-induced release of adrenocorticotrophic hormone (ACTH).[22] The F level in the body can be detected by monitoring the level in biological materials, such as serum, saliva, urine, and even feces. Here, serum has been in use as the preferred biological sample for steroid assay. Serum collection from individuals has its own drawbacks in terms of being an invasive approach. Saliva, on the other hand, has the advantage of being easily collected, without pain or special skill.[23] The disadvantages of the RIA system, and the high cost of commercial kits for routine use have encouraged us to establish our own system. Saliva EIA for F steroid is a difficult system to establish, as we find the presence of assay interfering substances in the saliva which can be overcome by heating and diluting the sample. The cross-reaction of the polyclonal antibody with different steroids, as in Fig. 1, is a greater challenge that requires the use of more specific monoclonal antibodies. The cross-reaction with 17-hydroxyprogesterone was the greatest problem but, owing to its low level in saliva (24 ± 2 pg/mL) in healthy male subjects,[24] we can assume the interference to be of minor consequence. However, in spite of the high rate of cross reaction, the recovery percentage and coefficient of variation, both intra- and inter-assay, was within an acceptable range as the EIA systems for steroid assay mentioned in earlier works. Comparing our established system with commercial kits purchased from Salimetrics® showed a strong correlation ($r = 0.996$). The established system was used to assay the saliva and serum levels of F hormone, which revealed our EIA system's ability to show the difference in the gender and circadian level difference[25] in F level and the relevancy of the saliva F level to serum free F level.

This established system of saliva F EIA is a modification of our successful assay system for various steroids which we have been using for over a decade.[26] This system provides a cheap and reliable alternative to saliva F assays. However, use of monoclonal antibodies (attempts underway) will provide even better results. In this aspect, the experiments conducted to test the system's sensitivity and repeatability was within an acceptable range. Thus, this system, with some more refinement, will prove to be a successful tool that could be used with preciseness to monitor the saliva F levels in the Taiwanese populace.

CONCLUSION

This experiment was an attempt to establish a saliva F EIA system which, though lacking in some aspect of preciseness, is sensitive enough to be comparable to a commercial kit. However, the use of monoclonal antibody (which we are working on currently) will sharply enhance the assay system. A stabilized EIA system for saliva steroids has been a challenge, due to many factors, ranging from low levels of steroids to interfering factors. Hence, we subjected our established system to many tests to assess its sensitivity and

reproducibility. The details of the established procedures and the subjected tests are explained herein. The sensitivity of the system at the low end was calculated to be 90 pg/mL. The intra- and inter-assay CV of the system were in the range of 1.7–4.6 and 17.8–18.7, respectively. The recovery rate of the added F in saliva was found to range from 75.8% to 100%, with better recovery at higher concentrations. The correlation of our established system with that of the kit obtained from Salimetrics® showed better results ($r = 0.996$) for the known level of F in the saliva sample, than for the unknown level ($r = 0.884$). In the same way, our established system, when used to assay the saliva and serum F levels from young Taiwanese volunteers, revealed detection of circadian variation as mentioned in the works of others. Likewise, the saliva F level was almost identical to the serum free F level; this indicated that saliva can be used to assess the status of bioavailable F in the serum. This system will definitely require refinement in terms of the antibody used, and the sample size used for the test. Nevertheless, the success in establishing the system signals a major breakthrough in saliva EIA research that will definitely have the potential of wider application in the Taiwanese context.

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