Radioimmunoassay for Cortisol in Pig Saliva and Serum

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An "in-house" radioimmunoassay (RIA) procedure utilizing commercial sources of antiserum and radiolabel was developed for the simultaneous measurement of cortisol in serum and saliva from swine. A commercial RIA kit (Incstar) for cortisol was used to validate the in-house procedure for serum measurements. The correlation coefficient (r) between the Incstar kit and the in-house method for serum cortisol was 0.94. The Incstar kit was modified to accommodate measurement of salivary cortisol concentrations and compared to the in-house procedure. The correlation between these procedures was $r = 0.81$ and best described by a logarithmic (Ln) curve. Salivary cortisol measures by the in-house RIA were significantly higher than those obtained from the modified Incstar kit ($p < 0.01$), a probable consequence of the different relative cross-reactions of the respective antisera to cortisone and a dilution effect on the kit standards.

Keywords: *Radioimmunoassay; salivary cortisol; pigs; stress*

INTRODUCTION

Reduced yield and degradation of meat quality are closely related to the exposure of animals to stressors commonly encountered in farming practice (Warris, 1985). Identification of stressors and the quantification of their biochemical and endocrinological effects are of continuing research interest. A fundamental practice in stress research is an evaluation of the status of the hypothalamic−pituitary−adrenal (HPA) axis. Increased activity of the HPA axis is indicative of a physiological response to stressors, and consequently, measurement of cortisol is a common component of stress research.

Conventionally, total cortisol concentrations are measured by immunoassay procedures in serum or plasma and, occasionally, urine. Blood and urine sampling regimes have several inherent problems. Obtaining a blood sample cannot be achieved without restraining the animal, thereby initiating a confounding activation of the HPA axis (Nyberg et al., 1988). The use of indwelling venous catheters overcomes this problem. Nevertheless, there are several constraints on their utility: The insertion of a canula is a skilled technique, they are often difficult to maintain over extended periods of time, their use is limited to studies involving small numbers of animals, and they can be extremely difficult to use in situations outside the environment of the animal's pen or barn.

In addition to the problems of obtaining blood samples, the measurement of cortisol in blood has physiological limitations. Cortisol in circulation is predominantly bound (90%) to cortisol-binding globulin (CBG) and albumin. The remaining 10% is in a "free" form, and it is this fraction that is available for uptake by target tissues. Under stress conditions the binding capacity of CBG becomes increasingly saturated, with a resulting disproportional increase in free cortisol. Thus, measurement of the total cortisol concentration in blood does not necessarily reflect the biologically active fraction of the hormone. Technical difficulties involved in the measurement of the free steroid hormone in blood makes this approach impractical for most routine purposes (Brien, 1980).

Cortisol concentrations in urine consist of the free hormone. However, urine is very difficult to collect, requiring special collection devices, severely limiting the number of animals that can be used in an experiment at any one time. Furthermore, cortisol levels in urine are dependent on urine volume. The concentration is often expressed as units of cortisol over a defined period, e.g., nanomoles per 24 h; consequently, the acute response of the HPA axis cannot be assessed.

Limitations of sampling and "biological validity" can be largely overcome by measuring cortisol in saliva. This sampling procedure is relatively easy to perform, does not require restraint of the animal, and can be achieved under conditions that are incompatible with blood and urine sampling, e.g. during transport. Cortisol enters saliva by a process of passive diffusion; the concentration is independent of flow rate and is a direct reflection of the free fraction in blood (Riad-Fahmy et al., 1982). Numerous studies have testified to the utility of salivary cortisol measurement for assessment of HPA axis status in various species (Read et al., 1982; Vining et al., 1983; Fell et al., 1985; Dathe et al., 1992; Greenwood and Shutt, 1992; Vincent and Michell, 1992; Malmud and Tabak, 1993). The utility of the measure in pigs is less well documented. Nevertheless, Cooper et al. (1989), Parrott et al. (1989), and Parrott and Misson (1989) have demonstrated good agreement between blood and saliva concentrations of cortisol in swine. In contrast, Blackshaw and Blackshaw (1989) reported "severe limitations" in the use of salivary cortisol due to the lack of a significant correlation between salivary and plasma concentrations. In a recent study, Cook et al. (1996) have shown excellent agreement between serum and salivary cortisol in response to adrenal stimulation by intravenous injection of 200 IU of adrenalcorticotrophic hormone (ACTH) and restraint by means of a nose-snare.

Minimally invasive sampling techniques are required for accurate assessment of endocrinological responses to stressors. We describe procedures for the collection of whole, mixed saliva from pigs and a RIA method for the simultaneous measurement of cortisol in serum and saliva. A commercially available RIA kit for cortisol was used to validate the serum assay. Simple modifications to the kit permitted measurement of cortisol concentrations in saliva.

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MATERIALS AND METHODS

Animals. Animals ($n = 6$) used in these experiments were York-Landrace cross female pigs of approximately 100 kg. They were housed in individual stalls of 3 m × 2.4 m. Animals were maintained on a balanced, barley-based diet, fed ad libitum (National Research Council, 1988). The pigs were housed and cared for in accordance with the Canadian Council on Animal Care Guidelines (1984). Housing temperatures were maintained at approximately 17 °C. Fluorescent lighting was on between 8:00 a.m. and 5:00 p.m. Water was available ad libitum via drinking spigots, and a bedding of straw was provided daily.

Sample Collection. Blood samples were collected via an indwelling ear-vein catheter, placed in situ as per the method of Schaefer et al. (1987). The catheter was secured with elastic tape to the back of the animal. The pigs were allowed to "normalize" for a period of 24 h before blood samples were drawn into plain glass tubes and allowed to clot before centrifugation and separation of serum. Samples were stored at −80 °C until required for assay.

Saliva samples were obtained on cotton-wool swabs, fixed to the ends of 1 m aluminum rods by means of rubber stoppers placed at either end of the swab. Pigs chewed on the rubber stoppers until the swabs were thoroughly soaked with saliva, a process that generally took <2 min and was often quicker than drawing the blood sample. The length of the rod was such that it was usually possible to obtain samples from outside the pen, thereby minimizing the disturbance to the animal. In a previous study the missed sample rate for saliva collection was <1%. After sample collection, the swab was placed in plastic syringes (5 mL capacity) which were fitted into conical-shaped, plastic centrifuge tubes (12 mL capacity) for transport to the laboratory. The samples were extracted from the cotton swabs by centrifugation at 1932*g* for 20 min. The volume of sample collected varied but was most often between 0.5 and 1 mL of saliva per swab. Food debris either remained on the swab or formed a pellet in the centrifuge tube. Samples were stored at −80 °C until required for assay.

Samples were from an experiment to investigate the efficacy of salivary cortisol for assessing the adrenal responses of swine to various stressors, including ACTH stimulation (Cook et al., 1996). Coste et al. (1994) has reported that the precision of hormone analysis is greatly increased for "peak" concentrations. Thus, the samples used in these experiments were selected because they covered a wide range of concentrations and had sufficient volume for analysis by all methods.

Commercial RIA (Incstar). A commercial method for the measurement of cortisol in serum samples was purchased from Incstar Corp., Still Water, MN. This method was the Clinical Assays GammaCoat Cortisol ^{125}I RIA kit. The kit utilizes a solid-phase antiserum coated to polypropylene tubes and a [^{125}I]cortisol label. The assay of cortisol in serum samples was as per the kit instructions.

The Incstar kit is claimed to be highly specific for cortisol. Endogenous steroids demonstrating significant cross-reactivities are 11-deoxycortisol (6.3%) and 17α-hydroxyprogesterone (1.2%). The cross-reactivity with cortisone was <0.1%.

The sensitivity of the assay was calculated according to the method of Rodbard (1978) and reported to be 0.21 μg dL^{-1}. Given a sample volume of 10 μL, this equates to a minimum detectable concentration of 5.8 nmol L^{-1} and is close to that of the in-house procedure.

The kit was adapted for measurement of cortisol in samples of saliva. This was achieved by adding assay buffer (100 μL) to the tubes containing standards. Saliva samples (100 μL) and 10 μL of "blank" standard were dispensed into the "sample" tubes. The amounts of label and incubation times were as per the assay protocol.

In-House Radioimmunoassay. The assay buffer was 0.5 M phosphate-buffered saline (pH 7.4) containing 0.1% gelatin (PBS/G). This buffer was used as the diluent for the antiserum, radiolabel, and dose−response curve standards.

Rabbit antiserum raised against cortisol-3-oxime bovine serum albumin was purchased from Endocrine Sciences, Calabasas Hills, CA. The antiserum has negligible cross-reactivity with most endogenous steroids with the exceptions of cortisone (30%), corticosterone (2.9%), desoxycorticosterone (4.5%), and 21-deoxycortisol (6.8%). Synthetic corticosteroids gave relative cross-reactivities of 0.1% (dexamethasone), 52% (prednisolone), and 26% (prednisone). The antiserum has a low cross-reaction with deoxycorticosterone (DOC) (<0.02%), and therefore a large dose of DOC is added to the antiserum to displace cortisol from CBG in direct assay systems. The reconstituted antiserum was used at a dilution of 1/5000.

The radiolabel was cortisol [1,2,6,7-^{3}H(N)], purchased from NEN DuPont, Mississauga, ON. The label was diluted in assay buffer to give approximately 15 000 counts min^{-1} 100 μL^{-1}.

Dose−response curve standards of hydrocortisone (Sigma Chemical Co., Mississauga, ON) were prepared in assay buffer. An initial stock solution of 1 mg mL^{-1} was prepared in ethanol. This stock solution was diluted with assay buffer to give a "top standard" of 1 ng 100 μL^{-1}. Further dilutions of the top standard were made to construct a standard curve ranging in concentration from 15 to 1000 pg 100 μL^{-1}.

Quality controls of high, medium, and low concentrations for the saliva assay were made in assay buffer according to a protocol similar to that for the standards. The quality controls for the serum assay were constructed by charcoal-stripping pig serum and spiking with known amounts of hydrocortisone.

Assay Protocol. The salivary cortisol assay involved dispensing, in duplicate, 100 μL of standards, quality controls, and samples into flint-glass culture tubes. Radiolabel (100 μL) of approximately 15 000 cpm was added to all tubes. The reagents were mixed and allowed to stand at room temperature for 30 min. Suitably diluted antiserum (100 μL) was added to all tubes except those designated to contain only radiolabel. The tubes were vortexed and incubated overnight at room temperature or for 3 h at 37 °C. Separation of the "bound" and "free" fractions was achieved by absorption of the free fraction with dextran-coated charcoal.

The assay for serum cortisol differed in that 10 μL of sample and quality controls were used. The consistency of volume and "matrix" was maintained by the addition of 10 μL of charcoal-stripped pig serum to the "standard" tubes and 100 μL of assay buffer to the quality control and sample tubes. Separation of the bound and free fractions was achieved by end-point precipitation of the bound fraction with 3 M ammonium sulfate.

These protocols permitted the measurement of cortisol in serum and saliva within the same assay. If saliva samples were to be included in a serum assay, 10 μL of stripped pig serum was added to the dispensed saliva sample. The protocol was as per the serum assay.

Logarithmic conversion of the standard concentrations plotted against counts yielded a straight line, and results for unknowns were interpolated from the resulting straight-line equation.

The sensitivity of the assay was calculated from 10 replicate standard curves and was taken as the dose equivalent to two standard deviations from zero. This definition and method of calculating sensitivity is the same as that used by the kit manufacturer and is generally regarded as the "standard" definition. We recognize that for most practical purposes a "reportable" minimal concentration should be derived from the precision profile, which is usually higher than that derived from the calculation of sensitivity. The sensitivity of the in-house assay was calculated to be 20 pg per assay tube, which equates to minimum detectable concentrations of 5.5 and 0.55 nmol L^{-1} for serum and saliva assays, respectively.

The intra-assay precision of the assays was demonstrated by the construction of the precision profiles for serum- and salivary-based analyses. The method for the construction of the precision profiles was a modification of that of Brown et al. (1957). Results from the assay of cortisol in a variety of experiments were combined to calculate the precision profiles (saliva, $n = 783$; serum, $n = 1144$). The mean results between single determinations were calculated and the data listed in numerically ascending order based on the mean values. Results were grouped into intervals and, after correction for duplicate determinations, the coefficient of variation in each of the intervals was calculated and plotted against the mean

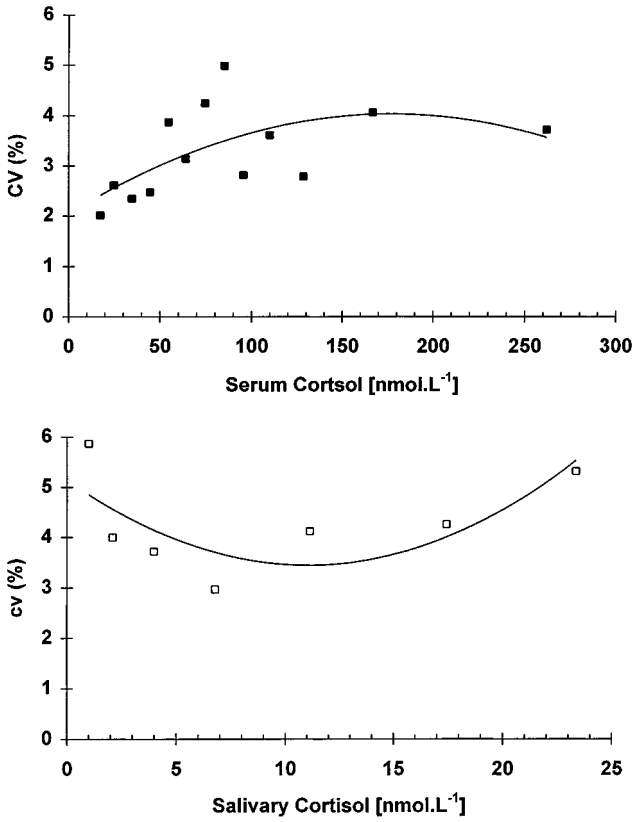

Figure 1. Intra-assay precision profiles of in-house RIA procedure for measurement of cortisol in (a) serum (■) (n = 1144) and (b) saliva (□) (n = 783).

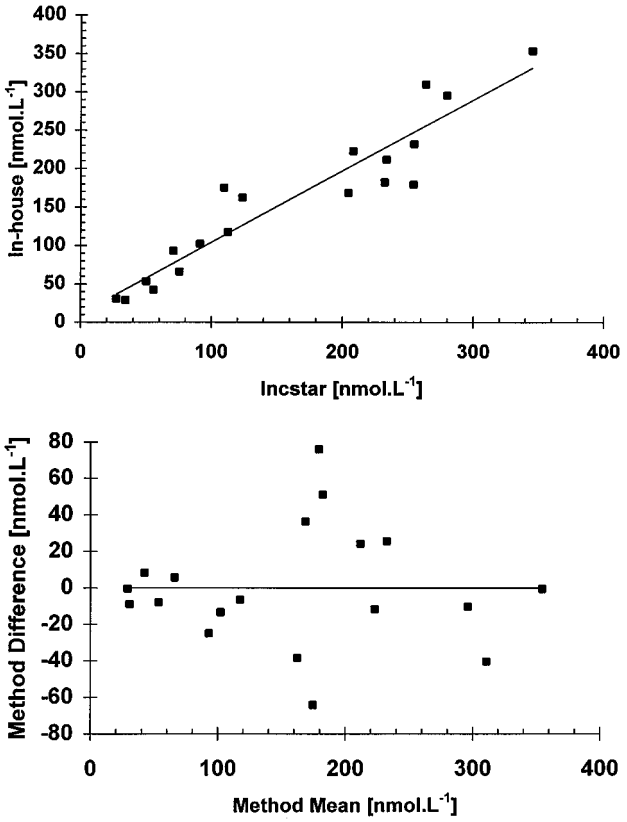

Figure 2. Cortisol concentrations in samples of pig serum (n = 19): (a) scatter diagram of Incstar RIA vs in-house RIA (line of best fit was derived from linear regression analysis); (b) scatter diagram of the differences between methods vs the method mean (line of best fit was derived from linear regression analysis).

value of the interval. Precision profiles for the serum and salivary cortisol assays are illustrated in Figure 1. The trendline was derived from a polynomial curve fit generated on Microsoft Excel (v 7). The imprecision of the serum assay was <5% for cortisol concentrations between 20 and 300 nmol L^{-1} and <6% for the saliva assay for concentrations between 1 and 25 nmol L^{-1}.

Inter-assay precision was calculated from replicate analyses (n = 15) of quality controls. The inter-assay coefficients of variation for serum quality controls at concentrations of 233, 78.5, and 26 nmol L^{-1} were 8.6%, 6.7%, and 6.6%, respectively. Similarly, for buffer quality controls at concentrations of 25.3, 8.6, and 2.7 nmol L^{-1}, the inter-assay coefficients of variation were 3.5%, 3.2%, and 6.5%, respectively.

Statistical Analyses. Scatter diagrams were used to illustrate the relationship between methods. The line of best fit was obtained from the curve fitting facility on Microsoft Excel (v 7). The curve of choice was that providing the highest correlation coefficient (r) between measures and an intercept that was closest to zero. Differences between mean concentrations, derived from different methods, were tested for statistical significance using Student's t test for paired data. An F test was used to test the variance in both measures.

RESULTS AND DISCUSSION

Methodological Comparisons for the Assay of Serum Cortisol. The range in concentrations was between 27 and 355 nmol L^{-1} and was similar to those reported in the literature (von Borell and Ladewig, 1989, 1992; von Borell and Hurnick, 1991; Mendl et al., 1993). Scatter diagrams illustrating the relationship between methods are presented in Figure 2. The line of best fit was included to enhance the interpretation of these relationships. The measurements of serum concentrations were in good agreement between RIA methods. Figure 2a demonstrates that these methods provided results that were closely associated and that a linear model most closely fitted the data. The regression equation that described this model was $Y = 0.9296X + 11.404$ (r = 0.9425), where Y = Incstar and X = in-house.

A plot (Figure 2b) of method differences vs method mean demonstrates that the differences between methods are not concentration dependent over the range 20−400 nmol L^{-1}, and this was confirmed by the correlation coefficient for the residual plot ($r^2 = 3E^{-29}$). The line of best fit from linear regression is included to illustrate the above.

A t test of the population means revealed that there were no significant differences ($p = 0.98$) between mean results obtained by the Incstar and in-house methods. Neither were there any significant differences ($p = 0.48$) in the F test for variance. Results from the in-house procedure were, on average, 1.03 times greater than those obtained by the Incstar method, which is probably a function of the higher cross-reactivities of the antiserum used for the in-house procedure. The high degree of correlation, linear regression, residual plot, and nonsignificant differences in t tests and F tests demonstrated the close agreement between RIA methodologies. The in-house method was therefore validated against the Incstar kit.

Methodological Comparisons for the Assay of Salivary Cortisol. Results obtained for the analysis of cortisol in saliva samples by both RIA methodologies are shown in Figure 3. In the absence of a definitive method for salivary cortisol measurement, it is impossible to judge which of the RIA procedures had the greater accuracy. On average, the ratio of in-house to Incstar results was 1.32, and a t test revealed a

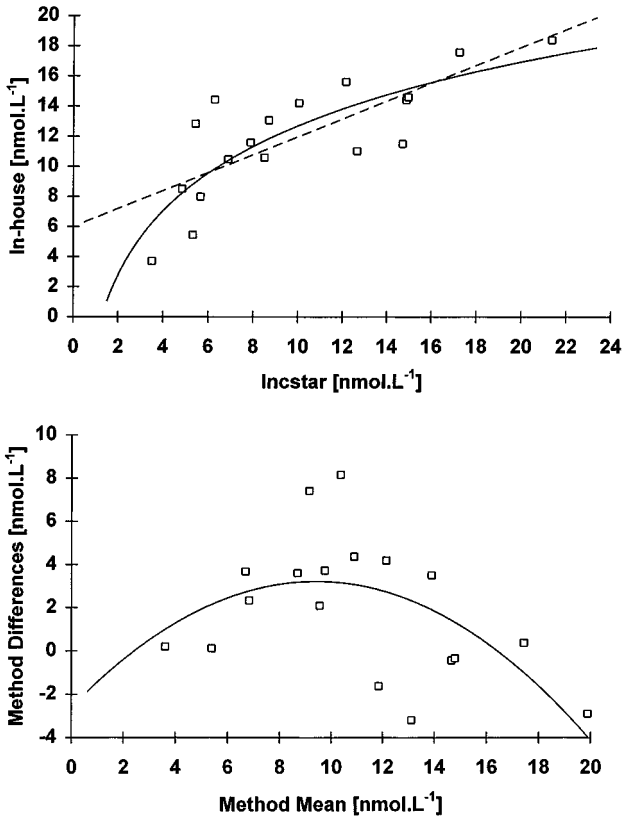

Figure 3. Cortisol concentrations in samples of pig saliva ($n = 18$): (a) scatter diagram of modified Incstar RIA vs in-house RIA [lines of fit were derived from linear regression analysis and a logarithmic (Ln) function generated by Excel (v 7)]; (b) scatter diagram of the differences between methods vs the method mean [line of fit was derived from a polynomial function generated by Excel (v 7)].

statistically significant difference ($p < 0.01$). However, both assays provided results that were within a range of 3.1−21.3 nmol L^{-1}, representing 5- and 5.8-fold ranges for the Incstar and in-house assays, respectively. The equations and correlation coefficients describing the relationship between salivary cortisol methods were $Y = 0.5996X + 5.9874$ ($r = 0.77$) and $Y = 6.1778\ \mathrm{Ln}(X) - 105101$ ($r = 0.81$) for linear and logarithmic (Ln) functions, respectively. Linear regression does not describe the relationship between these salivary methods as well as that for serum samples and was best described by a logarithmic (Ln) curve. Figure 3b demonstrates that the differences between these methods are concentration dependent and described by a polynomial curve fit. The correlation for the residuals was $r = 0.5747$ ($p < 0.01$), indicating that the differences between methods were concentration dependent.

Essentially, the same modification was made to both RIA procedures to accommodate the assay of salivary cortisol, i.e., a 1:10 dilution of the standards. It would therefore be expected that, given the linear relationship between the serum methods, the salivary cortisol analyses would also follow a linear regression. The standards used in the in-house RIA are the same for both the serum and saliva assays. The Incstar kit standards are "calibrated" against U.S.P. Cortisol Reference Standards. Thus, it is possible that the commercial method contains standards that vary slightly from the stated concentration in order to provide serum results in agreement with these reference standards. Consequently, a 1:10 dilution of the kit standards may distort the standard curve resulting in nonlinear regression.

Cortisol is converted to cortisone in the salivary gland, and the higher relative cross-reactivity of the in-house antiserum probably accounts for some of the discrepancy between methods. However, the effect of differential cross-reactivity is likely to be small. The concentration of cortisone in saliva required to give an apparent 30% higher cortisol level by the in-house procedure would need to be at least equal to that of cortisol. There is no evidence in pigs to suggest that this is the case. The Incstar kit is designed to measure cortisol concentrations in human serum and, as such, the most stable part of the standard curve coincides with cortisol concentrations most frequently encountered in "normal" human subjects under normal conditions, whereas the in-house RIA is optimized for cortisol measurement in pigs. Given that serum cortisol levels are lower in the pig and that salivary levels are approximately 10% of the serum concentration (Cook et al., 1996), it was perhaps optimistic to expect good linear agreement between these methods.

The blank effects of various strength solutions of pig feed were calculated to establish if contamination of the saliva samples with food had any effect on the assay. The apparent cortisol concentrations in these "blanks" ranged between 0.26 and 0.53 nmol L^{-1}. These values are below the sensitivity of the assay (0.55 nmol L^{-1}). Thus, food contamination of the saliva sample has no appreciable effect on the assay.

The material used for saliva collection may have significant effects on the assay. Foam rubber was originally used since this material is highly absorbent and easy to fix to the aluminum rods. However, depending on the density of the foam rubber, we discovered that this material had either significant blanking effects or stripped the sample of steroid content. Attempts to remove these effects were unsuccessful. Cotton-wool proved to be the best material for collection of a saliva sample. However, previous experience of saliva collection with cotton-wool swabs has shown that this may not always be the case, probably due to bleaching agents used in the manufacture of the cotton-wool interfering with the antibody−antigen reaction in the RIA. Trial and error may be required to find a material that is efficacious.

The obvious advantages of the in-house procedure is in terms of accuracy and the cost of analysis per sample. The cost of the Incstar method for serum cortisol was approximately 60% more per sample than that for the in-house method. This cost discrepancy increases if the Incstar kit is to be used for the measurement of salivary cortisol since adaptations to the kit require an extra blank calibrator. The concentration-dependent differences between the in-house salivary cortisol method and the modified kit suggests that the kit may be adaptable for measurement of the low concentrations in saliva but that the results may not be comparable to other methods. However, the adrenal response to different stressors may be assessed using a modified commercial kit provided there is consistency of use.

The results validate the in-house method for measurement of serum cortisol. The benefits of salivary cortisol analyses in terms of sampling, or biological validity of endocrine assessment, could be achieved with simple modifications to a commercially available kit for serum cortisol. However, the time taken to develop a simple, direct in-house RIA capable of simultaneous measurement of cortisol in serum and saliva may prove advantageous in terms of validity of measurement and

cost effectiveness. Furthermore, an in-house method is more readily adaptable to alternative sampling regimes that utilize small sample volumes such as capillary blood or blood-spot analyses.

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