Salivary cortisol on ROCHE Elecsys immunoassay system: pilot biological variation studies

Simon K. Chiu a, Christine P. Collier b, Albert F. Clark a, b, Katherine E. Wynn-Edwards c

a Department of Biochemistry, Queen’s University, Ontario, Canada
b Department of Pathology, Queen’s University, and Division of Clinical Laboratories, Kingston General Hospital, Ontario, Canada
c Department of Biology, Queen’s University, Ontario, Canada

Abstract

Objectives: Salivary analysis is a noninvasive alternative that may be more acceptable to patients, especially children. As such, it has the potential for incorporation into comprehensive, dynamic investigations of metabolic dysfunctions - a significant advancement over a single time point serum analysis. In this study, we wanted to determine if the serum cortisol assay on our routine immunoassay analyzer could reliably measure salivary cortisol concentrations. Because of potential fluctuations in salivary concentrations, we included a biologic variation study as a main facet of our preliminary method evaluation.

Design and Methods: Twenty-eight healthy individuals (12 males, 16 females) volunteered to provide 5 nonconsecutive first morning saliva samples over a two-week period. Samples were stored frozen at home until the completion of the study. Following thawing and centrifugation, cortisol was measured in batch mode for each set of participant samples on the ROCHE Elecsys. Biologic variation was determined following removal of outliers. A method comparison was performed with the DPC Coat-A-Count Cortisol assay following the recommended modifications for salivary analysis, and with the Salimetrics HS-Cortisol two-site monoclonal assay optimized for salivary cortisol.

Results: Mean salivary cortisol concentration was 20.4 nmol/L. Analytical variation (CV A = 3.8%), within-subject variation (CV I = 6.3%), between-subject variation (CV G = 20.5%), index of individuality (II = 0.36) and reference change value (RCV = 20.4%) were determined. A negative 40% proportional bias was observed on the Elecsys compared to the two methods that have already been optimized for salivary cortisol.

Conclusions: This study demonstrated that salivary cortisol can be reliably measured on a routine automated immunoassay analyzer such as the ROCHE Elecsys. This particular assay needs to be optimized at the low end of the standard curve for routine use with salivary samples. Based on the relatively small intra-individual variation and low index of individuality, reference change values are preferable to the use of population reference intervals for this assay. © 2003 The Canadian Society of Clinical Chemists. All rights reserved.

Keywords: Salivary cortisol; Biologic variation; Routine immunoassay analyzer; Roche Elecsys

Cortisol is used in the assessment of adrenal, pituitary, and hypothalamic function, and is especially important in the diagnoses of Cushing’s syndrome or disease and Addison’s disease. Prolonged exposure to high cortisol levels can lead to fatigue, high blood pressure, and hyperglycemia. Conversely, prolonged depression of cortisol concentrations can lead to fatigue, excessive weight loss, and muscle weakness. While total serum concentration is the primary measure following the administration of synacthen and dexamethasone, some centers include 24 h urine measurements for free cortisol in their protocols. More recently, several publications have indicated the usefulness of salivary free cortisol, especially with younger children [1,2,3]. In this study, we evaluated the ability of our automated cortisol immunoassay (ROCHE, Elecsys) to measure salivary cortisol in a study of the day to day biologic variation of morning samples from 28 normal volunteers.

The serum cortisol assay on the Elecsys 1010/2010 analyzers is a competitive polyclonal antibody immunoassay that employs a magnetic separation step followed by electrochemiluminescence quantitation (Roche Diagnostics, Laval, Quebec). This assay takes 18 min to run, uses 20 μL of sample, has a measurement range of 1.0 to 1750 nmol/L (0.04–63 μg/dL),
and a functional sensitivity (defined as CV<20%) of < 8.0 nmol/L (0.3 µg/dL). Routine internal quality control for this serum assay is currently performed at 415 nmol/L (CV<4.0%) and 775 nmol/L (CV<3.0%).

Twenty eight healthy, nonhospitalized volunteers (12 males/16 females; age 10–65 yr; median 23 yr) provided 5 nonconsecutive first morning samples over a two week period. They were provided with a “kit” in a self-sealing bag that contained a sheet with collection directions and five 12 × 75 mm capped polypropylene tubes marked to indicate the 5 mL required sample volume. The participants were directed to collect saliva immediately upon awakening and before eating or brushing their teeth. Instructions emphasized that they should “spit clear saliva and try to minimize the amount of mucous”. Samples were stored immediately in their home freezers (-20°C) until all five collections were completed (saliva methods reported in the literature, commonly include a freezing step to facilitate the breakdown of mucous before centrifugation). Following transportation to the laboratory, samples were stored at -20°C for a maximum of two weeks. Following thawing, they were vortex mixed for 30 s, and centrifuged at 2880 g for 10 min. The supernatant was removed and transferred to an Elecsys sample cup for analysis. For this study, we did not perform replicate analysis of the samples.

Investigation for outlier results for each participant’s set of samples was performed by comparing each participant’s standard deviation to the mean of all standard deviations ± 3 SD. One sample was excluded from a female participant based on this analysis and the SD was recalculated after this trimming procedure. To determine if any individual participant was an outlier from the group, we used Cochran’s variance test [4,5], which compared the participant’s individual variance contribution to the total variance (for 28 participants, no variance should exceed 0.145 at a probability of 95%). One male participant was excluded on this basis and is discussed below. The study summary statistics are thus presented for 27 participants (16 female, 11 male) and 134 data points.

The raw data for individual participants ranged from 6.4 to 39.1 nmol/L and are illustrated by gender and age in Fig. 1. Frequency plots of the participants’ mean salivary cortisol concentrations and their %CVs are shown in Fig. 2. A probability plot was used to confirm that the distribution of the means was Gaussian (not shown). Mean salivary cortisol concentrations ranged from 12.7 to 28.8 nmol/L for individuals, with an overall mean of 20.4 nmol/L. There was no statistical difference between women (mean = 19.7 nmol/L) and men (mean = 21.4 nmol/L) (t_{df=25} = 1.01, p = 0.32). The %CV for individual participants ranged from 6.1% to 48.4%. (Fig. 3.)

Table 1 summarizes the analytical variation for the salivary controls (CV_{A}), the within-subject variation (CV_{I}), the between-subject variation (CV_{G}), the index of individuality (II), and the reference change value (RCV) that would need to be exceeded if serial samples were to be significantly different with a probability of less than 0.05 [4,6].

Two main limitations of this study should be noted. The first is that as a pilot study, the testing was done in a few batches only. As a result the total number of control results used for the determination of CV_{A} is small (n = 10 for within day CV_{A} and n = 6 for between day CV_{A}). This estimate needs to be confirmed in future studies and should ultimately be within the quality specifications for salivary cortisol based on CV_{I} (i.e., a desirable CV_{A} of less than 3.2% (0.5 CV_{I}) or a minimal CV_{A} of less than 4.7% (0.75 CV_{I})) [6]. We anticipate that this precision is possible. A second limitation of our study is related to the lack of replicates (a cost decision made based on the pilot nature of the study). The estimates of the variables in Table 1 would be more reliable if replicate analysis had been performed for each sample and then used for the calculation of CV_{A} instead of estimating CV_{A} from our limited control data.

The intra-individual variation (CV_{I}) was 6.3% and the inter-individual variation (CV_{G}) was 20.5%, resulting in an index of individuality (II) of 0.36. In comparison, serum cortisol is reported to have a CV_{I} of 20.9% and a CV_{G} of 45.6%, for an II of 0.45 [6]. Population-based reference intervals are not of high clinically utility for tests with low indexes of individuality (II<0.6) [6]. This is because the range of values for a particular
subject will only span a small portion of the population reference interval and thus a significant change could occur within the population reference interval and not be detected. A more appropriate measure is “reference change value” (RCV) which determines whether a change in results is significant based on analytical and within-individual variations [6].

In this study, one participant had salivary cortisol concentrations which were elevated by 20-fold compared to the results for the other volunteers (139, 422, 704, 701 nmol/L; mean = 492 nmol/L and CV = 55%). The results for this participant were excluded from overall statistical analysis. Repeat saliva collections 6 months later demonstrated similar results (62, 284, 322, 371, 865 nmol/L; mean = 381 nmol/L and CV = 76%). Subsequent simultaneous collection of fasting saliva and serum yielded results of 457 nmol/L for saliva and 278 nmol/L for serum (reference range: 170–800 nmol/L). This participant was a 64 yr old male who was on Pravastatin, Hydrochlorothiazide and Diltiazem and Vitamin E. These drugs are not known to interfere with cortisol assays or to affect in vivo cortisol metabolism. Furthermore, hemolysis was not visible, ruling out blood contamination. Thus, at the present time, we have no explanation for the high salivary cortisol values in this participant.

Reports on a wide variety of uses for salivary cortisol measurement have increased recently as investigators appreciate that salivary sampling is fast, convenient, safe and reliable [2,3,7–10]. It is important to ensure that the bias between methods is minimal so that results obtained by different methods can be correlated. In a recent report, Raff et al. [9] found that calibrator measurements returned expected results for both their Diagnostic Systems Laboratories (DSL) and Diagnostic Products Corporation (DPC) methods. However in their recovery studies, they found that the DSL method overestimated saliva concentrations by 19% while the DPC results were much closer to expected results. In an initial validation of the accuracy of the Elecsys assay for salivary cortisol, we also compared it with the DPC method (Coat-A-Count Cortisol Assay modified for salivary analysis, Diagnostic Products Corporation, Los Angeles, California) and with the Salimetrics HS-Cortisol two-site monoclonal assay which is specifically produced for only salivary cortisol measurement (Salimetrics LLC, State College, Pennsylvania). Analysis of the calibrators from these two assays on the Elecsys produced expected calibrator concentrations (Elecsys = 0.986 DPC for 6 calibrators ranging from 0.0–138.0 nmol/L, and Elecsys = 0.926 Salimetrics for 7 calibrators ranging from 0.0–49.7 nmol/L). However, when 18 pediatric salivary samples that had been previously measured by DPC and Salimetrics assays (DPC = 0.986 DPC, R² = 0.72) were measured on the Elecsys a proportional bias of 40% was observed (Elecsys = 0.646 DPC, Elecsys = 0.594 Salimetrics). As the usefulness of salivary cortisol analysis becomes more evident, manufacturer standardization and optimization in the low salivary cortisol concentration ranges should be addressed as soon as possible to facilitate research and protocols across methods.

In summary, this study demonstrates that salivary cortisol can be reliably measured on a routine automated immunoassay analyzer such as the ROCHE Elecsys. As salivary cortisol testing is noninvasive, comprehensive investigation of cortisol homeostasis can be performed easily in both adults and children. Our pilot biologic variation study demonstrated that within individual variation is relatively small.
for salivary cortisol and suggested that interpretation of results would be more effective if reference change values were used instead of population reference intervals.

**Acknowledgments**

We would like to thank Dr. CG Fraser, (Ninewells Hospital and Medical School, Dundee, Scotland) and Mr. A. Day (Biostatistician, Kingston General Hospital) for their review and suggestions on our manuscript. We also appreciate the support of Roche Diagnostics in supplying the cortisol kits for this study.

**References**


