Letter to the Editor

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Evaluation of an automated chemiluminescent immunoassay for salivary cortisol measurement. Utility in the diagnosis of Cushing's syndrome

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To the Editor,

Saliva is a biological fluid easy to obtain with non-invasive procedures in a stress-free way compared with the serum collection for the determination of hormone levels [1]. It has been suggested that measurement of salivary cortisol levels is more appropriate for the clinical assessment of adrenocortical function. In fact, determination of late-night salivary cortisol concentration is recommended for the screening of patients with suspected Cushing's syndrome (CS) [2]. Automated systems have a rapid turn-around time on a large number of samples and have demonstrated high analytical accuracy. Given that salivary cortisol levels are a valuable indicator of the hypothalamic-pituitary-adrenocortical axis activity [3], its determination by automated systems may allow a broader use of this diagnostic tool [4, 5].

The aim of this study was to validate an automated chemiluminescent immunoassay (CLIA), Siemens Immulite 2000® analyzer (Gwynedd, UK), for the measurement of salivary cortisol and to compare it with the electrochemiluminescent immunoassay (ECLIA), Roche Cobas e-411 (Mannheim, Germany), which has been validated by the manufacturer for the measurement of salivary cortisol. To validate the assay we evaluated limit of blank (LOB), limit of detection (LOD) and limit of quantification (LOQ), precision, linearity, recovery and we performed the method comparison. We have also determined the cut-off level for salivary cortisol by CLIA in CS diagnosis in an adult population.

To evaluate the analytical performance of CLIA and compare it with ECLIA, 50 saliva samples were collected from healthy volunteers at 8 am (27 males and 23 females; body mass index, 25.5 ± 1.5 kg/m^2). Analysis of salivary cortisol cut-off values were performed on late-night salivary samples (11 pm) obtained from 38 healthy subjects (24 males and 14 females; body mass index, 24.9 ± 1.9 kg/m^2, subjects who presented serious underlying medical conditions were excluded from the study) and from 21 newly diagnosed CS patients (6 males and 15 females; body mass index, 28.7 ± 2.2 kg/m^2) without medication. All samples were collected through passive drool directly into sterile plastic tubes of single use. Saliva donors did not brush their teeth, smoke, eat, or drink anything except for water for 2 h before testing. Volunteers and patients with fresh cuts or abrasions within the oral cavity were excluded.

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Morning samples were immediately stored at –20 °C until analysis and late-night samples were first preserved at 4 °C and immediately stored at –20 °C the next morning. Once thawed, samples were centrifuged at 1500 *g* for 10 min at room temperature and supernatants were used for cortisol determinations. Volume samples required for cortisol determinations by CLIA and ECLIA were 10 µL and 20 µL, per reaction, respectively. This study was approved by the Ethics Committee at the Hospital de Clínicas "General José de San Martín" (according to the Helsinki Declaration for medical studies).

Salivary cortisol levels were measured with CLIA method with minor modifications. We performed a new calibration curve with dilutions of cortisol calibrator solutions in a range of 2.0–100 nmol/L using CLIA's kit diluent recommended by the manufacturer. Calibration solutions were obtained from RIA kit, Immunotech® Beckman Coulter (catalog number: IM1841), which were calibrated against the reference preparation ERM®-DA192 and 193. Logit-log calibration curves were constructed with values from calibrators in counts per second (cps) and sample values were interpolated into this straight line to determine cortisol concentrations in nmol/L.

To validate CLIA for salivary cortisol, LOB, LOD, and LOQ were assessed according to the EP-17A protocol [6]. LOB was 0.9 nmol/L, established as the 95th percentile from repeated non-parametric measurements (60 times) of a blank sample (CLIA's kit diluent). No significant differences were found in the calibration curve when CLIA's diluent or saliva were used. The slopes for the assay with CLIA's diluent and saliva were –1.6674 (95% CI, –2.3728 to –0.9620; $y = 1.994 - 1.6674x$; $r = -0.990$) and –1.1177 (95% CI, –1.5498 to –0.6855; $y = 1.794 - 1.1177x$; $r = -0.992$), respectively. Data distribution used for LOD calculation was nonparametric (Shapiro-Wilks $p < 0.05$), and no significant differences were found between variances (F test, $p < 0.05$). The obtained LOD was 2.0 nmol/L, which was established from repeated measurements of three sample pools of saliva with concentrations between LOB and four times LOB. The LOQ value was 3.4 nmol/L, determined from a precision profile using four saliva samples of different concentrations (Figure 1). In the precision assay, the coefficient of variation intra-assay for concentrations of 11.5 and 20 nmol/L were 8.9% and 6.0%, respectively, and the coefficients of variation inter-assay for the same concentrations were 9.9% and 9.4%, respectively.

To evaluate linearity in the salivary matrix, serial dilutions of a cortisol standard solution were performed using saliva as diluent (cortisol concentration in the matrix was lower than 2 nmol/L). This assay was linear between 2.0 and 100 nmol/L.

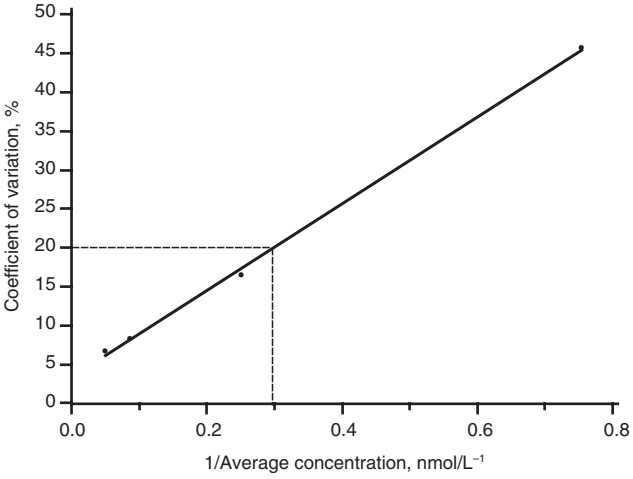

Figure 1: Imprecision profile of salivary cortisol by regression analysis after linearization (x-axis, inverse of salivary cortisol average concentration; y-axis, inter-assay variation evaluated in quadruplicate throughout a 7-day study).
The LOQ was defined as the concentration that results in a variation coefficient of 20% (dotted line).

In the recovery test, three saliva samples, with a known cortisol concentration, were supplemented with 9, 21 or 47 nmol/L of cortisol and the mean analytical recovery were 107.2%, 103.5% and 97.8%, respectively. Each determination was performed in triplicates.

To evaluate method comparison, salivary cortisol from 50 volunteers was measured by CLIA and ECLIA. Salivary cortisol concentrations determined by CLIA (median, 6.0 nmol/L; range, 6.0–9.0 nmol/L) were significantly lower compared to ECLIA values (median, 8.5 nmol/L; range, 8.5–10.7 nmol/L, $p < 0.0001$ Mann-Whitney test). The correlation between both methods was significant (Spearman's rank correlation coefficient: 0.656; $p < 0.0001$). A Bland-Altman plot was performed to evaluate the concordance of the two immunoassays; the bias between both methods (CLIA minus ECLIA values) was –1.9 nmol/L. Although the correlation was not optimal, an appropriate concordance was achieved and the disparities could be attributed to differences in the antibodies used in each immunoassay.

In this study, to optimize the diagnostic performance of the test, CLSI international standards were applied to determine the LOQ before establishing the cut-off value. This parameter was evaluated using a receiver operator characteristic (ROC) analysis curve (MedCalc Statistical Software v12.7.7, Ostend, Belgium) performed with 59 late-night salivary samples. CLIA showed a good diagnostic performance (area under the curve, 0.990, $p < 0.0001$, Figure 2A). In addition, the best cut-off value for CLIA

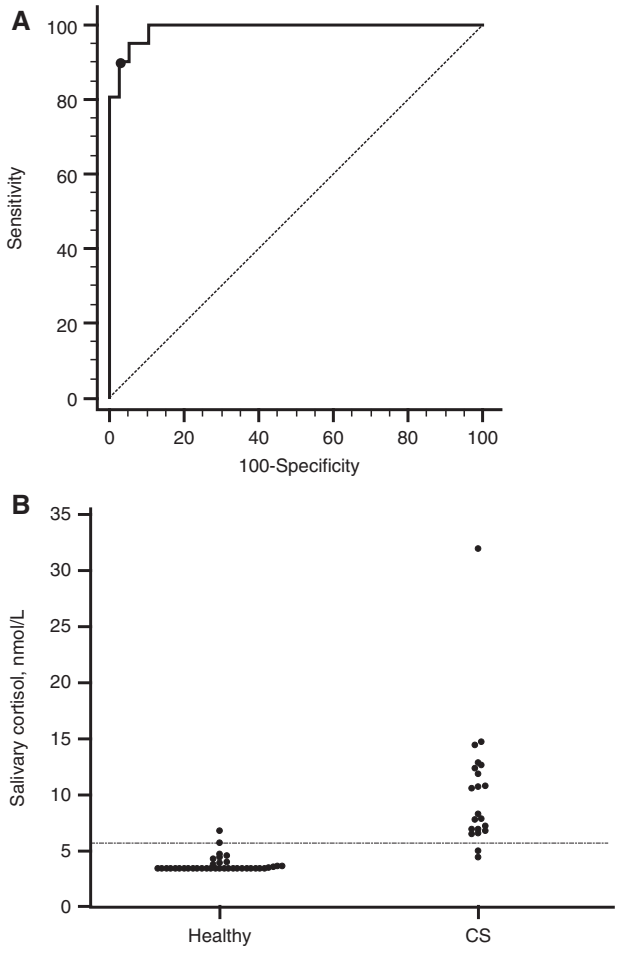

Figure 2: Salivary cortisol analysis.
(A) ROC analysis of late-night salivary cortisol (11 pm) measured by CLIA (bold line). The optimal cut-off value obtained was 5.7 nmol/L (filled point) with 90.5% sensitivity (95% CI, 69.6–98.8) and 97.4% specificity (95% CI, 86.2–99.9). (B) Individual data points for midnight salivary cortisol values by CLIA of healthy patients (healthy: n = 38; median, 3.4 nmol/L; interquartile range, 3.4–3.7 nmol/L) and patients with Cushing's syndrome (CS: n = 21, median, 8.3 nmol/L and interquartile range 6.9–12.5 nmol/L), $p < 0.0001$, Mann-Whitney test for independent samples. A broken horizontal line represents the cut-off point value obtained.

obtained from the ROC curve was 5.7 nmol/L, with an optimal sensitivity and specificity of 90.5% and 97.4%, respectively (Figure 2B).

The International Endocrine Society recommends late-night salivary cortisol determinations as a first line screening test for CS [2]. Several studies have shown that it is necessary to establish cut-off values for salivary cortisol measurement in CS diagnosis, according to the method used [7]. The cut-off point (5.7 nmol/L) was not so different to those reported by others in previous studies with immunoassays [8]. However, the diversity in the cut-off values could be attributed to differences in the methods used and the population studied, as well as the source of variability that affect the determination of cortisol [8, 9].

In summary, this study shows the validation of a chemiluminescent automated method for the measurement of salivary cortisol that presents convenient analytical performance in values close to cut-off level. Considering the emerging potential roles of salivary cortisol measurement (stress biomarker, congenital adrenal hyperplasia or adrenal insufficiency biomarker) [2], the method described here can be easily used in clinical laboratories for diagnostic purposes since it is relatively simple to perform very sensitive, and has a satisfactory turn-around time. Moreover, this method offers the possibility to analyze samples as they are received and help clinicians to make more timely clinical decisions.

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