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Multicenter performance evaluation of a second generation cortisol assay

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Abstract

Background: Untreated disorders of the adrenocortical system, such as Cushing's or Addison's disease, can be fatal, and accurate quantification of a patient's cortisol levels is vital for diagnosis. The objective of this study was to assess the analytical performance of a new fully-automated Elecsys® Cortisol II assay (second generation) to measure cortisol levels in serum and saliva.
Methods: Four European investigational sites assessed the intermediate precision and reproducibility of the Cortisol II assay (Roche Diagnostics) under routine conditions. Method comparisons of the Cortisol II assay vs. liquid chromatography-tandem mass spectrometry (LC-MS/MS), the gold standard for cortisol measurement, were performed. Cortisol reference ranges from three US sites were determined using samples from self-reported healthy individuals.
Results: The coefficients of variation (CVs) for repeatability, intermediate precision, and reproducibility for serum samples were ≤ 2.6%, ≤ 5.8%, and ≤ 9.5%, respectively, and for saliva were ≤ 4.4% and ≤ 10.9%, and ≤ 11.4%, respectively. Agreement between the Cortisol II assay and LC-MS/MS in serum samples was close, with a slope of 1.02 and an intercept of 4.473 nmol/L. Reference range samples were collected from healthy individuals (n = 300) and serum morning cortisol concentrations (5–95th percentile) were 166.1–507 nmol/L and afternoon concentrations were 73.8–291 nmol/L. Morning, afternoon, and midnight saliva concentrations (95th percentile) were 20.3, 6.94, and 7.56 nmol/L, respectively.
Conclusions: The Cortisol II assay had good precision over the entire measuring range and had excellent agreement with LC-MS/MS. This test was found suitable for routine diagnostic application and will be valuable for the diagnosis of adrenocortical diseases.

Keywords: assay; cortisol; immunoassay; method comparison; performance evaluation.

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Introduction

Diseases of the adreno-cortisol system – such as Cushing's disease and Addison's disease – are rather rare conditions [1–3]; life-threatening on the one hand but treatable on the other [4]. Consequently, a straightforward diagnostic approach is of essential importance, and accurate quantification of cortisol levels plays a key role in the diagnosis of patients with suspected disease [5]. Cortisol levels are often measured from a patient's serum or plasma, which reflects total cortisol, both free and bound. However, in patients with liver disease, those receiving estrogen treatment or with critical illness, total serum cortisol levels may be difficult to interpret because of the variation in binding proteins. Recently, quantification of cortisol in saliva has become more widely used since it is

considered of equal diagnostic relevance as serum but can be obtained more easily and non-invasively [6, 7]. Salivary cortisol is free (i.e. not bound) and is useful in patients with serum results that are challenging to interpret [8, 9]. Very low salivary cortisol concentrations are typically found in healthy individuals (< 8 nmol/L at midnight) [10, 11]. Therefore, accurate test results in the lower cortisol ranges are needed because this is where reproducibility is currently reduced but where diagnostic test decisions are often made.

Automated cortisol immunoassays are commercially available, which have acceptable precision and are widely used not only because of their high performance but also because of their speed, simplicity, and cost-effectiveness [12]. One major limitation of immunoassays is their specificity with interference of structurally similar compounds, for example endogenous steroids such as cortisone, or other steroid drugs such as prednisolone or dexamethasone [13, 14]. In addition, different immunoassays show high inter-assay variation [12, 15].

Gas chromatography-tandem mass spectrometry (GC-MS/MS) is traditionally used as a reference method for serum cortisol quantification. The introduction of LC-MS/MS allowed the use of isotope dilution mass spectrometry also in routine testing; however, the application of this technology is still very demanding and limited to very few laboratories at present [16–20] with The Reference Institute for Bioanalytics (RfB; Bonn, Germany) reporting only 2.7% (14/513) laboratories using LC-MS/MS in their last survey [21]. Consequently, immunoassays are still the mainstay of cortisol testing worldwide [22].

The first generation Elecsys® Cortisol assay (Roche Diagnostics) was introduced in April 2000 to measure cortisol levels in serum, plasma, urine, and saliva and has been used for the quantification of cortisol in clinical laboratories worldwide [23–26]. In order to improve the specificity of cortisol measurement, a novel second-generation cortisol assay (Elecsys® Cortisol II assay; Roche Diagnostics) has been developed. Poly-clonal cortisol-specific antibodies in the first generation assay were switched to more specific monoclonal antibodies in the new assay. Both the first- and second-generation assays measure the total cortisol fraction in serum and the free cortisol in saliva [22, 23, 27]. Furthermore, the new test generation is traceable to a GC-MS/MS-based reference method according to the concept of an unbroken chain of traceability in clinical laboratory testing. The objective of this study was to assess the analytical performance of the Cortisol II assay under field conditions in a multicenter performance evaluation study.

Materials and methods

Study sites and instruments

The technical evaluation of the Cortisol II assay was performed between June and November 2014 at four European investigational sites: three in Germany (Munich, Heidelberg, and Leipzig) and one in Belgium (Ghent). All sites used **cobas e** 411 analyzers for the Cortisol II assay experiments presented in this study. In addition to a **cobas e** 411 system, the Leipzig site utilized a **cobas e** 601 analyzer.

Assay design

The Elecsys® Cortisol II assay is the second generation assay (Roche Diagnostics GmbH, Mannheim, Germany) and can be used to quantify cortisol levels in serum, plasma (Li-heparin, K_2-K_3-EDTA), or saliva. Briefly, the sample is incubated with a biotinylated cortisol antibody along with a ruthenium complex labeled cortisol derivative to form an immune complex. Streptavidin-coated microparticles are added and the complex becomes bound to the solid phase via a biotin/streptavidin interaction. Once aspirated, the microparticles are magnetically captured onto the surface of an electrode within a measuring cell, a voltage is applied with an electrochemiluminescence signal as the readout. The sample volume required is 10 µL, the assay measuring range is 1.5–1750.0 nmol/L, and the entire procedure takes a total of 18 min [27]. The assay is calibrated using the Cortisol CalSet (Roche Diagnostics) and has been standardized to the Institute for Reference Materials and Measurements (IRMM; Geel, Belgium)/IFCC-451 panel (ID-GC/MS) for serum [28].

Ethics statement

The investigation and sample collection sites adhered to the International Conference on Harmonization guideline for Good Clinical Practice and conducted the study in accordance with the Declaration of Helsinki (revised version). The study was approved by the Local Ethics Committees of the participating sites. All individuals participating in the reference range study signed an informed consent agreement.

Sample storage and handling

The control samples for the precision experiments were stored at – 80 °C at Roche Diagnostics GmbH before being shipped to the study sites. At the sites, the samples were stored at – 20 or – 80 °C. For the method comparison experiments, leftover, anonymized serum or plasma and saliva samples from daily routine were used. These were stored at – 20 °C until measurements, for a maximum of 12 weeks [29].

For the reference range assessments, serum or plasma was collected using a 3.5-mL Vacutainer® Serum Separation Tube (SST). A serum sample was preferred, but alternatively, Li-heparin-plasma/K_2,-K_3-EDTA plasma was used. Saliva was obtained using a cotton swab Salivette® device (Sarstedt, Germany). Samples were processed within 24 h of donation. For the samples obtained at midnight, these were stored at 2–8 °C and delivered to the collection sites in the morning hours, no later than 24 h after taken. After collection, all samples were stored at 2–8 °C for a maximum of 3 days then frozen at – 70 °C

at the collection facilities. The handling of the saliva samples was in line with a previous study into the long-term stability of saliva cortisol [29]. At the end of the collection process, frozen specimens were shipped on dry ice to the Roche Diagnostics Indianapolis site in a thermally insulated container and were received at the destination in a frozen state. Good Laboratory Practices and national regulations for the shipping of samples were strictly followed. Upon receipt, the samples were stored at − 80 °C before being shipped to the study testing site. The samples at the study testing sites were stored at − 70 °C until testing occurred. Samples that were not handled, stored, or shipped according to this procedure were excluded from the analysis.

Cross-reactivity

The cross-reactivity of the Cortisol II and Cortisol (generation 1) assays was determined using two human serum samples, one of which was spiked with potential cross-reactant compounds. The cortisol concentration of the samples was approximately 165.5 nmol/L (60 µg/L) and 551.7 nmol/L (200 µg/L) (conversion factor µg/L × 2.7586 = nmol/L). The spiked and non-spiked samples were tested in duplicate and the difference from the non-spiked sample represented the analyte concentration simulated by cross-reaction. The percentage of cross-reaction was calculated using the following formula:

$$\text{Cross-reactivity (\%)} = \frac{100\times \text{ simulated cortisol concentration}}{\text{spiked cross-reactant concentration}}$$

The following cross-reactants were assessed: 6-α-methylprednisolone (0.1 mg/L), cortisone (10 mg/L), dexamethasone (10 mg/L), prednisolone (0.1 mg/L), and prednisone (10 mg/L). The concentrations used to test the cross-reactivity were chosen based on those previously used to test the cross-reactivity of the first generation Cortisol assay [26]. These tests, in part, followed the Clinical and Laboratory Standards Institute Evaluation Protocol (CLSI EP) 07-A2 Guideline [30].

Precision and accuracy

Assay precision was evaluated using quality control samples which were provided by Roche Diagnostics. These were the serum control samples Preci Control Universal 1 and 2 (PC U1 and PC U2). In addition, five lots of human sample pools (HSP 01–05) spiked with cortisol were used: HSP 01 was a saliva control and HSP 02–05 were serum controls. The concentration of the control samples were determined by Roche and were defined as the target concentration. All precision experiments were performed in compliance with the CLSI EP 05-A3 guideline [31]. A minimum of 30 specimens (same matrix as the precision samples) were included in every run between the precision determinations to simulate normal laboratory conditions.

Repeatability (intra-assay) and intermediate (inter-assay) precision experiments were performed in a four-fold determination on the same day over 21 days. The resulting mean concentration and the coefficients of variation (CVs, %) were calculated. Reproducibility was evaluated at each site with five human sample pools (HSP

Table 1: LC-MS/MS methods used for method comparison at the three study sites.

	Ghent	Leipzig	Munich
Instrument	LC Shimadzu MS ABSciex Triple Quad 5500	Serum: QTRAP® 6500 Saliva: QTRAP® 5500	Water Acquity UPLC Waters Xevo TQ-S
Ionization mode	APCI +	APCI +	ESI+
Mass transition	Cortisol: 363.2/121.1 Cortisol-d4: 367.2/121.1	Cortisol: 363/121 Cortisol-d4: 367/121	Cortisol 363/121 Cortisol-d3 366/121
Sample preparation	Liquid/liquid extraction (serum and saliva)	Protein precipitation plus on-line SPE Serum: 100 µL Saliva: 50 µL	Protein precipitation plus on-line SPE Sample volume: 100 µL
Calibrators	In-house	Serum: MassChrom® Steroids (Chromsystems, Munich, Germany) Saliva: in-house	In-house
QC samples	NIST Reference material 971 UK NEQAS for Steroid Hormones	Serum: MassChrom® Steroids (Chromsystems, Munich, Germany) and in-house pooled control Saliva: in-house	Serum pools (in-house) PC Saliva 1 (Roche) Generic QCs (in-house)
Intermediate precision	8%	Serum: 2.9%–4.9% Saliva: 4.5%–5.1%	Serum pools: 1.3%–4.9% PC Saliva 1: 3.6% Generic QCs: 5.8%–6.3%
Reference	Fiers et al. [32] Janssens et al. [33]	Serum: Ceglarek et al. [34], Gaudl et al. [35] Saliva: Bae et al. [36]	Suhr et al. [37]
Validation	CLSI C57 [38] and C62-A [39] guidelines	According to the FDA guidance [40], EU guideline [41], and ICH guideline [42]	Based on the EMA guideline [41]

ACPI, atmospheric pressure chemical ionization; ESI, electrospray ionization; NIST, National Institute of Standards and Technology; NEQAS, National External Quality Assessment Scheme; QC, quality control; SPE, solid phase extraction; TQ-S, tandem quadrupole mass spectrometer; UPLC, ultra performance liquid chromatography.

01–05), two QC materials (PCU 1 and 2) on a five-fold determination over 5 days. This resulted in 175 analyses per site, using three different lots of reagent from all four sites.

The reference serum samples (HM 3/13A and HM 3/13B) from the RfB proficiency testing program were analyzed at each of the four European sites. The RfB runs a reference measurement procedure, which is done according to an International Organization for Standardization (ISO) standard by accredited laboratories only listed by the Joint Committee of Traceability in Laboratory Medicine (JCTLM), and is based on GC-MS. The assigned values for HM3/13A and HM3/13B were obtained by the RfB using this reference measurement procedure. The samples were tested in a three-fold determination on three separate days.

Method comparison

Leftover, anonymized serum or plasma and saliva samples from daily routine were used for the method comparison experiments. The Cortisol II assay was compared with the Cortisol assay (generation 1) on either the **cobas e** 411 or the **cobas e** 601 systems at the four study sites. The Cortisol II assay was also compared with in-house isotope dilution LC-MS/MS assays. The LC-MS/MS methods are summarized in Table 1. The general use of cortisol of LC-MS/MS in endocrine testing has also been published elsewhere [17].

Reference range assessment

The reference range samples were collected from healthy individuals at three US sites between June and December 2014. The collection sites were Medical Research Center (Spartanburg, SC, USA), Premier Research (Austin, TX, USA), and Quest Research Institute (Bingham Farms, MI, USA). Testing was performed between October and December 2014 at the Washington University School of Medicine, Core Laboratory for Clinical Studies (St. Louis, MO, USA).

Key inclusion criteria were age ≥ 21 years, self-reported healthy status, no infectious disease or symptoms in the prior 8 weeks, and a minimum volume of 1 mL each of serum/plasma or saliva was required. Individuals were excluded if they were pregnant, lactating, using oral contraceptives, receiving medication with cortisone/cortisol or hormone replacement therapy, or unable to read or understand and sign the informed consent/assent form.

In total, a minimum of 120 evaluable individuals were required to be recruited in order to calculate the 5th to 95th/2.5th to 97.5th (serum/plasma) and the 95th/97.5th (saliva) percentiles. For determination of the Cortisol II assay reference ranges, the population was required to have a distribution of race/ethnicities of 40% African American/40% Caucasian/20% Hispanic, in a female to male ratio of approximately 1:1. Morning samples (n = 150 minimum required) were collected between 6 and 10 a.m. independent of waking time. Afternoon samples (n = 150 minimum required) were collected between 4 and 8 p.m. in order to have a closer view of circadian rhythm of cortisol, decreasing over the period a.m.-p.m.-midnight. Midnight saliva samples were allowed to be collected at 12 p.m. ± 30 min at a fixed time independent from the bedtime.

Data management and statistical analysis

The output from the Cortisol II assay was directly captured on the Windows-based computer-aided evaluation (WinCAEv), a Code of Federal Regulations (CFR) 21 Part 11 compliant electronic data capture software [43]. The WinCAEv software was run from a laptop attached to the **cobas e** 411 and **cobas e** 601 analyzers. Repeatability, intermediate precision, and reproducibility were calculated using the statistical programs SAS (version 9.3) and R (version 3.0.1, additive package VCA version 1.0.6), in compliance with the CLSI EP05-A3 Guideline [31].

An outlier analysis was performed on the precision samples at the end of the study: the outliers results were defined according to the recommendations of the CLSI EP05-A3 guidelines [31]. Based on these, it was permitted to reject at most two results due to an outlier, which was equal to one part in a 21-day study. This was applied to each precision sample and each 21-day experiment. The recovery of the target value for each sample based on the median of all determinations was calculated for accuracy experiments. Passing-Bablok regression analysis and Pearson correlation coefficients were used to assess the correlation of the method comparison studies in compliance with CLSI EP09-A3 [44].

The reference range analysis was performed using SAS software version 9.3 and R version 3.0.1. For serum and plasma samples, the 2.5th, 5th, 95th, and 95th percentiles were calculated. The 95th and 97th percentiles were reported for saliva samples. The limit of quantitation (LoQ) and the limit of detection (LoD) of the Cortisol II assay were calculated based on the CLSI EP17-A2 Guideline [45]. It is important that the LoD and LoQ were the same for both the serum/plasma and saliva samples: for standardization of the assay, both serum/plasma and saliva samples were used to ensure no matrix effect. The LoD and LoQ results of the assay were not recorded as part of the study.

Results

Cross-reactivity

To assess the specificity of the new Cortisol II assay vs. the first generation one, compounds with similar structures to cortisol were measured on both assays. The new Cortisol II assay monoclonal antibody had an improved cross-reactivity profile compared with the Cortisol assay (generation 1) for 6-α-methylprednisolone, dexamethasone, and prednisolone (Table 2). Cross-reactivity levels were higher with the second generation assay vs. the first in the measurement of cortisone and prednisone, but both were still very low (≤ 6.58%).

Precision and accuracy

Across the four European sites, the repeatability CVs for the HSP 01–05 samples (excluding outliers) ranged from 0.8% to 4.4% (Table 3). For the PC U1 and PC U2 samples, the repeatability CVs were between 1.3% and 2.2%.

The intermediate precision CVs for the HSP 02–05 serum samples ranged from 2.1% to 5.8%, but were

Table 2: Cross-reactivity of the first and second generation cortisol assay antibodies.

Substance	Concentration, mg/L[a]	Cortisol (generation 1)	Cortisol II
		Cross-reactivity, %	Cross-reactivity, %
6-α-Methylprednisolone	0.1	389	12
Cortisone	10	0.30	6.58
Dexamethasone	10	0.08	ND
Prednisolone	0.1	171	7.98
Prednisone	10	0.28	2.23

[a]Concentration of substance added; ND, non-detectable.

Table 3: Precision results of the Cortisol II assay.

Sample	Type	n	Target conc., nmol/L[a]	Mean measured conc., nmol/L[b]	Repeatability CV, %[c]	Intermediate precision CV, %[c]	Reproducibility CV, %
HSP 01	Saliva	414	8.43	8.44	2.8–4.4	5.0–10.9	11.4
HSP 02	Serum	416	96.0	99.7	1.1–2.2	2.3–4.2	6.8
HSP 03	Serum	418	489.0	482	0.8–2.3	2.1–4.8	7.5
HSP 04	Serum	418	982.0	966	1.1–2.5	2.7–5.6	9.5
HSP 05	Serum	336[d]	1605.0	1611	1.8–2.6	2.5–5.8	5.8
PC U1	Serum	418	304	310	1.4–2.2	1.9–4.1	4.6
PC U2	Serum	420	734	736	1.3–2.2	2.0–4.8	5.1

[a]The target concentration was provided by the manufacturer; [b]mean concentration measured by the sites; [c]range of CVs observed across the four study sites; [d]data from one site not included in this analysis due to samples initially being tested without dilution – retesting not possible due to stability date being exceeded. Conversion of units: μg/dL × 27.59 = nmol/L; nmol/L × 0.0362 = μg/dL.

Table 4: Accuracy results of the Cortisol II assay.

RfB sample	Sample type	Target, nmol/L	Accuracy per center range, %	CV of accuracy results across all centers, %
HM 3/13A	Serum	667	−8.3 to +1.4	−0.9
HM 3/13B	Serum	365	−5.9 to +0.1	−0.7

higher for the saliva sample (HSP 01, 5.0%–10.9%). The intermediate precision CVs for the two PC control samples were between 1.9% and 4.8%. Reproducibility CVs across the four laboratories ranged from 5.8% to 9.5% for the HSP 02–05 serum samples, and 11.4% for the saliva HSP 01 sample. The reproducibility CVs were 4.6%–5.1% for the PC U1 and PC U2 control samples, respectively.

A test of the accuracy of the assay was performed by comparison with GC-MS, based on proficiency testing samples specified by the RfB. Deviation from the GC-MS specified target concentrations of the proficiency testing serum cortisol samples were ≤10% (Table 4). CVs for the Cortisol II assay with the HM3/13A and HM3/13B GC-MS reference serum samples were −4.5% and −3.7%, respectively.

Method comparisons

For the method comparison studies, the serum samples covered a measuring range of 1.7–1735.0 nmol/L and the saliva samples from 1.5 to 209.5 nmol/L. The Passing-Bablok regression analysis for the Cortisol II assay and the Cortisol assay (generation 1) with serum samples (n = 541) resulted in a slope of 0.76 [95% confidence intervals (CI), 0.74–0.77] and an intercept of 10.3 nmol/L (95% CI, 6.09–15.53) (Figure 1A). The correlation coefficient was 0.968. Bland-Altman analysis showed a combined mean bias of −91.11 nmol/L (±2 SD, −238.91 to 56.69) across all sites (Figure 1B). For saliva samples (n = 404), the slope agreement for Cortisol II vs. the Cortisol assay (generation 1) was 1.21 (95% CI, 1.17–1.25) and the intercept was −5.5 nmol/L (95% CI, −5.97 to −5.01), with a correlation coefficient of 0.992 (Figure 1C). The Bland-Altman difference plots of the saliva samples on the Cortisol II vs. Cortisol (generation 1) assays revealed a mean bias of −2.72 nmol/L (±2 SD, −9.94 to 4.51; Figure 1D).

The Cortisol II assay was also compared with LC-MS/MS, the current gold standard for cortisol measurement. For serum samples (n = 405), the agreement between the Cortisol II assay and LC-MS/MS was high, with a slope of 1.02 (95% CI, 1.00–1.04), an intercept of 4.473

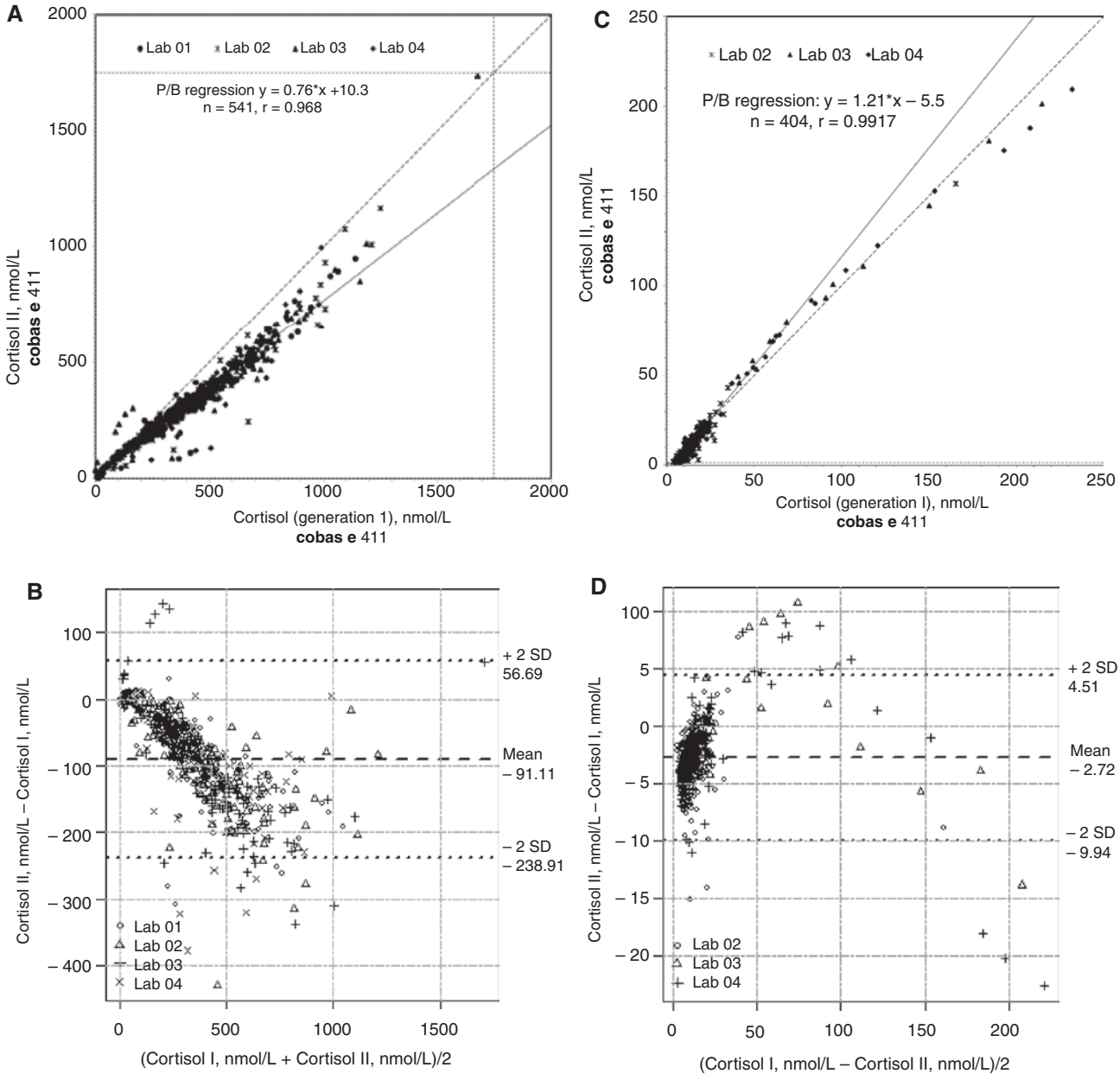

Figure 1: Method comparison results and Bland-Altman plots of the Cortisol assay vs. the Cortisol II assay in serum or plasma samples (A) and (B), and saliva samples (C) and (D).

nmol/L (95% CI, 0.94–9.45), and a correlation coefficient of 0.9863 (Figure 2A). The mean bias for serum samples measured on the Cortisol II assay compared with LC-MS/MS was 14.55 nmol/L (±2 SD, – 50.84 to 79.93; Figure 2B). The relationship between the Cortisol II assay vs. LC-MS/MS for saliva samples (n = 253) was given with a slope of 1.134 (95% CI, 1.10–1.16) and an intercept of 0.825 nmol/L (95% CI, 0.67–1.06) and a correlation coefficient of 0.9930 (Figure 2C). For these comparisons, the mean bias was 2.56 nmol/L (±2 SD, – 5.54 to 10.65; Figure 2D).

Reference ranges

To calculate the references ranges, a total of 894 saliva and 596 serum samples were collected from 300 self-reported healthy individuals and cortisol levels were measured using the Cortisol II assay (Table 5). Serum morning cortisol concentrations (5th–95th percentile) were 166.1–507 nmol/L and afternoon concentrations were 73.8–291 nmol/L. Morning, afternoon, and midnight saliva cortisol concentrations (95th percentile) were 20.3, 6.94, and 7.56 nmol/L, respectively. For

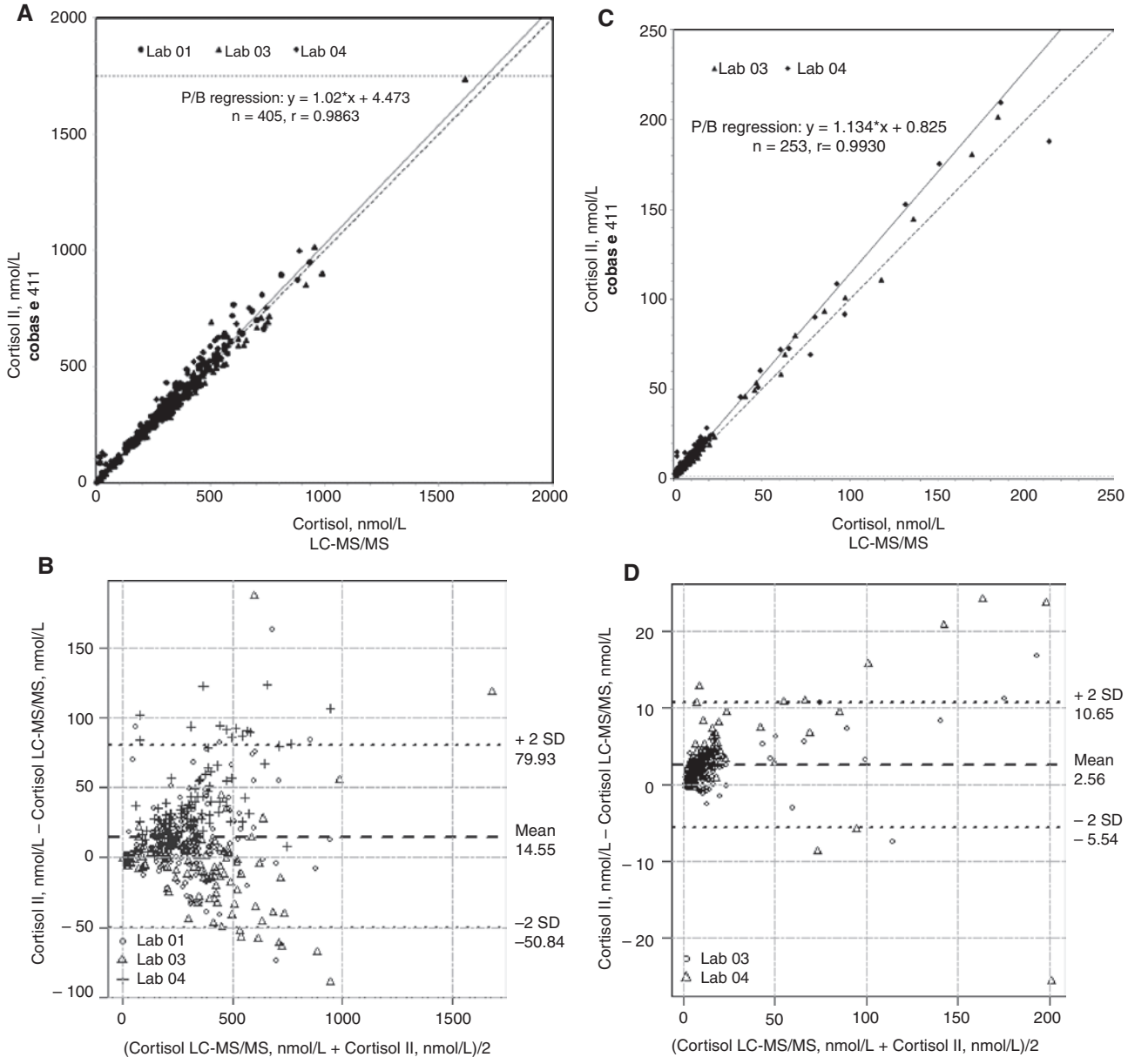

Figure 2: Method comparison results and Bland-Altman plots of the Cortisol II assay vs. LC-MS/MS in serum or plasma samples (A) and (B) and saliva samples (C) and (D).

midnight salivary cortisol, the median values were below the measuring range of the assay [LoD (3.0 nmol/L) and LoQ (1.5 nmol/L)] and the majority of individuals levels were below the LoD (81.6%) and the LoQ (61.5%). No statistically significant difference was observed between males and females for the serum or saliva samples.

Discussion

We here report the results of a performance evaluation study of a novel serum and salivary cortisol assay and, since serum and saliva cortisol measurements are key in the work-up of suspected adrenocortical diseases, these results are of clinical significance. The particular strength of this study was its multi-center and multi-lot design that aimed to simulate the long-term diagnostic application of this test in endocrine care. A main finding was that the between-laboratory and between-production lot reproducibility was roughly 10% in the typical concentration range of salivary cortisol and roughly 6%–10% for serum cortisol. According to the concept of critical difference, this allows to distinguish a true cortisol concentration of a diagnostic sample of 11.3 nmol/L (upper limit of the

Table 5: Cortisol II assay reference range values from serum or plasma and saliva of healthy individuals.

	n	Male/ female, n	Median, nmol/L	5th–95th percentile, nmol/L	2.5th–97.5th percentile, nmol/L	n (%) below LOQ[a]	n (%) below LOD[a]
Serum or plasma a.m.	296	144/152	302.7	166.1–507.3	132.9–537.0	0	0
Serum or plasma p.m.	300	146/154	160.6	73.8–291.5	68.2–327.0	0	0
				95th percentile, nmol/L	97.5th percentile, nmol/L		
Saliva a.m.	297	144/153	8.0	20.3	24.1	24 (8.1)	5 (1.7)
Saliva p.m.	298	146/152	2.7	6.94	9.65	171 (57.4)	75 (25.2)
Saliva midnight	299	146/153	< MR	7.56	11.3	244 (81.6)	184 (61.5)

A.m., 06:00–10:00; p.m., 16:00–22:00; midnight, 00:00 ± 30 min; MR, measuring range; [a]the lower limit of the reference range is defined by the LoQ (3.0 nmol/L) and by the LoD (1.5 nmol/L).

normal range observed in this study for midnight salivary cortisol) from 14.7 nmol/L with > 95% probability.

Despite being the gold standard, LC-MS/MS is still quite demanding and the complexity of the entire system makes routine measurement a very substantial challenge and it is still limited to very few laboratories [16–21]. An immunoassay offers a simpler approach in contrast. Compared to the first generation Cortisol assay, we observed improved agreement of results with LC-MS/MS, which currently represents the gold standard for cortisol measurement in the clinical laboratory. This improvement may be due to both the application of a more specific monoclonal antibody and the specification of calibrator concentrations which were now based on a GC-MS reference method [28]. Close agreement with LC-MS/MS was observed for a large series of serum and saliva samples, as well as good recovery of the RfB proficiency testing samples, demonstrated high accuracy of the study's assay.

Daytime serum and saliva cortisol concentrations were studied in a large cohort of self-reported healthy adults to address the need for assay-specific cut-offs. The cut-offs varied depending on the time of day and whether serum or saliva was measured (Table 5). Of note, the majority of the saliva midnight samples were below the LoQ (3.0 nmol/L) and the LoD (1.5 nmol/L) of the assay compared to the morning and afternoon samples. However, the LoD and LoQ of the assay was not investigated as part of this study and was the manufacturer's in-house data. It is important to state that the daytime data should not be used in a diagnostic approach in patients with suspected Cushing's syndrome. These patients typically have normal serum cortisol concentration in the morning and the most important method to exclude Cushing's syndrome is the dexamethasone suppression test. Alternatively, late-night cortisol secretion can be tested to exclude autonomous cortisol secretion in Cushing's syndrome due to excess adrenocorticotropic hormone synthesis by hypophyseal adenoma or extra-hypophyseal tumors as a paraneoplastic syndrome or excess cortisol secretion by adrenal adenomas. In this respect, the midnight salivary cortisol concentrations observed with the Cortisol II assay in the 299 healthy individuals are of substantial relevance in the context of this study. In this population, the 97.5th percentile was 11.3 nmol/L. This is slightly higher than reported for the first generation Cortisol assay (8.9 nmol/L) [25]. However, it is still recommended that each laboratory should determine and apply their own assay specific cut-offs in the diagnostic work-up of adrenocortical diseases when salivary cortisol is used since no conclusive system of between assay standardization of this emerging analyte in endocrine care has been achieved so far [46]. This is in contrast to serum cortisol measurement, which has a rather close between-assay agreement, as was observed in proficiency testing programs [47].

In summary, for the Cortisol II assay, we observed a degree of between-laboratory and between-production lot reproducibility and agreement with several assays of higher metrological order that we consider compatible with the diagnostic use of this assay. The Cortisol II assay will be beneficial to endocrinologists in assessing patients with adreno-cortisol disorders.

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