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# Inter-method comparison of salivary cortisol measurement

### Methodenvergleich der Cortisolmessung im Speichel

#### Abstract

**Background:** Salivary cortisol is increasingly used in the diagnostic work-up of suspected Cushing's disease as well as in stress research. In this study, the agreement of different methods for salivary cortisol measurement was assessed.

**Methods:** Saliva samples from five healthy volunteers were distributed to three routine clinical chemistry laboratories. As a reference, all samples were also analyzed using liquid chromatography-tandem mass spectrometry involving stable isotope labeled cortisol for internal standardization in our laboratory.

**Results:** All immunometric routine methods substantially overestimated salivary cortisol concentrations by approximately 50%. The agreement between these immunoassays was moderate with between-method coefficients of variation of up to 36% for individual samples.

**Conclusions:** Standardization of salivary cortisol measurement requires substantial improvement, in particular by implementation of proficiency testing schemes, a defined reference method, and reference materials. Interpretation of salivary cortisol data in psycho-neuroendocrinological research has to consider the inappropriate level of standardization realized for salivary cortisol measurement to date.

**Keywords:** immunoassay; liquid chromatography-tandem mass spectrometry (LC-MS/MS); method comparison; salivary cortisol; standardization; stress.

#### Zusammenfassung

**Einleitung:** Die Bestimmung von Cortisol im Speichel wird immer häufiger für die Diagnostik des Hypercortisolismus (Morbus Cushing) herangezogen und findet außerdem Einsatz in der Stressforschung. In dieser Arbeit wird die Vergleichbarkeit von verschiedenen Methoden der Speichelcortisolbestimmung überprüft. **Methoden:** Speichelproben von fünf gesunden Freiwilligen wurden an drei klinische Routinelaboratorien versandt. Als Referenz wurden die Proben im eigenen Labor mittels Flüssigchromatographie-Tandem-Massenspektrometrie vermessen, wobei als Interner Standard stabilisotopenmarkiertes Cortisol verwendet wurde.

**Ergebnisse:** Alle immunometrischen Routinemethoden überschätzten die Speichelcortisolkonzentration erheblich um ca. 50%. Die Übereinstimmung zwischen den Immunoassays war mittelmäßig, mit Variationskoeffizienten einer individuellen Probe innerhalb der Methoden von bis zu 36%.

**Schlussfolgerung:** Die Standardisierung der Bestimmung von Cortisol im Speichel muss deutlich verbessert werden, insbesondere durch die Einführung von Ringversuchen, durch eine definierte Referenzmethode und durch Referenzmaterialien. In der Psycho-Neuroendokrinologischen Forschung muss bei der Interpretation von Speichelcortisoldaten die ungenügende Standardisierung hinsichtlich der Speichelcortisol-Bestimmung berücksichtig werden.

**Schlüsselwörter:** Immunoassay; LC-MS/MS; Methoden-vergleich; Speichelcortisol; Standardisierung; Stress.

# Introduction

Salivary cortisol measurement allows non-invasive monitoring of the adrenocortical system and has, therefore,

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become a key tool in stress research during recent years [1]. In clinical endocrinology, salivary cortisol measurement is now recommended as a first line test in the diagnostic work-up of suspected hypercortisolism [2]. Several immunoassays for salivary cortisol measurement are commercially available and many commercial laboratories offer this test. However, salivary cortisol measurement is technically demanding and has important drawbacks that should be recognized. In contrast to established standard clinical chemistry analytes, no reference measurement system is implemented for salivary cortisol, no reference method is accepted, no reference material is available, and external quality assessment by proficiency testing is not implemented so far by independent organizations in Europe. Furthermore, immunoassays can be affected by cross-reactivity from other steroids (e.g., cortisone, corticosterone,  $6\beta$ -hydroxycortisol, prednisolone) [3].

Indeed, earlier studies have found substantial systematic between-method bias for salivary cortisol measurement in the context of endocrine diagnostic testing. Respective reports have mainly focused on the lowest concentration range of late night samples, which is relevant to exclude Cushing's disease [4, 5] and less on the entire range of physiological salivary cortisol concentrations as a relevant parameter for stress research [6, 7]; methods of higher metrological level were applied inconsistently. Consequently, there is uncertainty as to the commutability of research results related to absolute salivary cortisol concentrations in stress research. Therefore, the aim of our study was to re-assess the agreement between different routine salivary cortisol methods implemented in standard endocrinological laboratories and to compare the results with a highly specific, state-of-the-art mass spectrometric method as a reference.

## Materials and methods

After informed consent, saliva samples were obtained from five healthy volunteers using a commercially available sampling device (neutral cotton swab, Salivette<sup>®</sup>, Sarstedt, Nümbrecht, Germany). Sampling was done early in the morning (07:00–08:00 h) and late at night (22:00–23:00 h) to obtain a total of ten samples. Two swabs were used in each sampling to achieve at least 1 mL of saliva. The procedure was approved by the Institutional Review Board. The samples were anonymized and each sample was divided into five aliquots.

Aliquots of the ten samples were sent to two commercial laboratories by regular postal service as suggested in routine endocrinological testing. In one laboratory, an enzyme linked immunosorbent assay (ELISA) was used (Cortisol Saliva Elisa, IBL International GmbH, Hamburg, Germany); in the second laboratory, a radioimmunoassay (RIA) was used (Cortisol Coat-A-Count RIA Kit, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany). One aliquot of each sample was analyzed in the authors' laboratory using an automated immunoassay system (Cobas e 411, Roche Diagnostics GmbH, Mannheim, Germany) employing electro-chemiluminescence technology (ECLIA). This was done after storing the samples for 48 h at ambient temperature (approx. 22°C) in order to apply similar pre-analytical conditions for all analyses. One further aliquot from each sample was analyzed using isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Waters Acquity UPLC and Waters Xevo TQ-S system configuration, Waters, Milford, USA) as a reference. Three-fold deuterated cortisol was used for internal standardization and solvent extraction with dichloromethane was performed for sample preparation. The LC-MS/MS method and its specifications are described in online supplemental data.

## Results

The cortisol results of the ten study samples, which were obtained using the three different immunometric methods, are displayed in Table 1. The cortisol results were correlated using the algorithm described by Passing

Table 1 Salivary cortisol results obtained for the ten study samples.

	Sample	Salivary cortisol concentration, $\mu g/L$				CV for immunometric	CV for all analytical
		ECLIA	ELISA	RIA	LC-MS/MS	methods, %	systems, %
Late-night salivary samples	1	1.23	1.20	0.90	0.30	16.3	47.4
	2	1.25	1.00	0.70	0.27	27.8	52.1
	3	1.96	1.20	1.00	0.34	36.5	59.1
	4	3.56	3.20	2.40	1.70	19.4	30.6
	5	1.15	0.90	0.70	0.28	24.6	48.8
Morning salivary samples	6	3.59	4.60	4.40	2.97	12.8	19.4
	7	5.19	6.90	6.10	3.82	14.2	24.1
	8	3.89	2.70	2.30	1.28	27.8	42.4
	9	5.05	4.40	3.80	2.29	14.2	30.4
	10	3.72	3.90	3.80	2.03	2.4	26.5
Mean (n=10)		3.06	3.00	2.61	1.53		
Median (n=10)		3.57	2.95	2.35	1.49		

Bereitgestellt von | Bayerische Staatsbibliothek Angemeldet Heruntergeladen am | 05.11.15 09:21 and Bablok and the following regression equations, confidence intervals (CIs) of the slope and the intercept, as well as the correlation coefficients were calculated:

#### ECLIA vs. ELISA:

ELISA=1.44 (95% CI: 0.86–2.09)×ECLIA–1.12 (95% CI: –3.39 to 0.11); r=0.912

ECLIA vs. RIA:

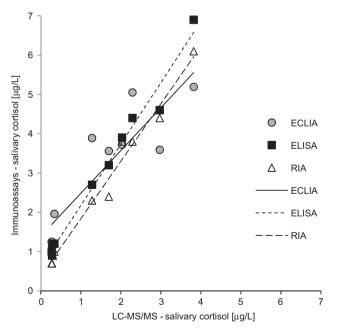
RIA=1.21 (95% CI: 0.68–1.59)×ECLIA–0.75 (95% CI: –2.12 to 0.04); r=0.893

#### ELISA vs. RIA:

RIA=0.92 (95% CI: 0.87–1.03)×ELISA-0.19 (95% CI: -0.35 to -0.04); r=0.993

The ratio between the highest and lowest results obtained from the three different immunoassays for individual samples ranged from 1.0 to 2.0. The coefficient of variations (CV) observed for the three immunometric methods ranged from 2.4% to 36.5% for individual samples (Table 1).

In comparison with all immunometric tests, substantially lower cortisol results were found with LC-MS/MS (Table 1). The CVs observed for all four analytical systems ranged from 19.4% to 59.1% for individual samples (Table 1). In Figure 1, the Passing and Bablok regression of the salivary cortisol results reported by three immunoassay systems related to LC-MS/MS as a reference is shown and



**Figure 1** Passing and Bablok regression of salivary cortisol results reported by three immunoassay systems related to LC-MS/MS as a reference.

the following regression equations, CI of the slope and the intercept, as well as the correlation coefficients were calculated:

LC-MS/MS vs. ECLIA: ECLIA=1.41 (95% CI: 0.82–2.06)×LC-MS/MS+0.86 (95% CI: 0.46–1.87); r=0.886

LC-MS/MS vs. ELISA: ELISA=1.64 (95% CI: 1.48–1.74)×LC-MS/MS+0.59 (95% CI: 0.42–0.73); r=0.991

LC-MS/MS vs. RIA: RIA=1.50 (95% CI: 1.29–1.74)×LC-MS/MS+0.38 (95% CI: 0.14–0.56); r=0.991

## Discussion

In a comparison study of salivary cortisol involving three routine immunometric methods, we observed moderate correlation and between-method agreement. The ratio of lowest to highest immunoassay results reached a factor of two for individual samples, and between-method CVs for individual samples ranged up to 36%. All immunoassay results were substantially higher compared with the results obtained by LC-MS/MS. Because LC-MS/MS with isotope dilution internal standardization is a highly specific and accurate method, this observation demonstrates a substantial overestimation of salivary cortisol by all immunometric methods studied.

The reason for this finding is most likely a pronounced cross-reactivity of immunometric cortisol assays with inactive cortisone. One essential pathway of cortisol inactivation is based on the conversion to biologically inactive cortisone by 11β-hydroxysteroid dehydrogenase type II [8]. The local activity of this enzyme in mineralocorticoid-sensitive tissues is essential to confer specificity towards aldosterone. Whereas in serum the concentration of cortisol to cortisone is approximately 10:1, in urine and saliva the concentration of cortisone is typically approximately two-fold higher compared with cortisol [9]. Because the molecular structure of cortisol differs from cortisone only in two hydrogen atoms, it is not surprising that immuno-assays with their intrinsically limited specificity are prone to cross-reactivity with cortisone.

Our observation is in line with previously published articles demonstrating substantial bias between salivary cortisol assays [4–7]. Consequently, the use of assay-specific cut-off concentrations is recommended for salivary cortisol assessment in the context of endocrine testing, for example, rule-out of Cushing's disease [2, 3].

These observations can be ascribed to a sub-optimum level of standardization and quality assurance realized for salivary cortisol measurement, including the following drawbacks. In contrast to the vast majority of routine clinical chemistry analytes, for salivary cortisol proficiency testing schemes have not been implemented so far by independent organizations in Europe. Quality control materials for comprehensive internal quality assessment of salivary cortisol measurement are not commercially available from independent sources. Furthermore, a common requirement for current clinical chemistry assays is the traceability of routine methods to reference materials, which is not realized for salivary cortisol. Such reference materials are usually manufactured and provided to the diagnostic industry for assay standardization, for example, by the US Institute of Standardization (National Institute of Standards and Technology, NIST). The target analyte concentration in such reference materials has to be specified by a mass spectrometric reference method. Reference methods are institutionally accredited, for example, by the Joint Committee for Traceability in Laboratory Medicine (JCTLM; www.bipm.org/en/committees/jc/jctlm/).

Recently, Miller et al. [7] compared the most widely used immunoassays with LC-MS/MS as a reference using a substantial number of samples (n=195) and similarly poor agreement between assays was observed. Applying a structural equation modeling framework, the authors tried to decompose systematic assay variance. They observed non-linear relations between immunoassays and LC-MS/ MS, which the authors attributed to immunoassay crossreactivity with saliva matrix components. To enable the comparison of salivary cortisol results from different methods, the authors provided guidelines to convert cortisol concentrations into comparable reference values. However, owing to differential cross-reactivity of immunoassays as the probable most essential problem of salivary cortisol measurement, this strategy seems questionable.

Salivary cortisol concentrations are approximately onetenth of serum concentrations, and salivary cortisol assays are simply the unmodified application of serum cortisol assays to the matrix saliva. With respect to protein content and pattern, as well as to steroid hormone pattern, this matrix profoundly differs from serum. Therefore, the chainof-traceability as realized for serum cortisol measurement cannot be conferred to salivary cortisol measurement.

Scientists in the field of stress research should be aware of the methodological limitations of salivary cortisol measurement. This is particularly the case when absolute salivary cortisol results (as for cut-off values) are reported (instead of relative descriptions, such as incremental factors).

LC-MS/MS becomes implemented in a continuously growing number of endocrinological laboratories. This technology realizes very high analytical specificity - similar to gas chromatography-mass spectrometry (GC-MS). In contrast to GC-MS, this technology is more robust and easier to handle [10]. Thus, this technology is increasingly looked upon as the gold standard for small molecule quantification in endocrinology. Indeed, the widespread application of LC-MS/MS has the potential to realize harmonization of salivary cortisol measurement, which can hardly be achieved with immunoassays. Whereas immunometric assays for small molecule analytes are very prone to poorly defined matrix interference and reagent lot-to-lot variation, LC-MS/MS can obviate practically all impacts of individual matrix factors by application of the principle of isotope dilution internal standardization. This enables the implementation of method-independent reference ranges, as realized for serum steroid hormones [11].

# Conclusions

In summary, we have found that salivary cortisol results are highly method-dependent at present and consistency of observations employing different assays is a critical issue. Although concurring results have been published over the past years, there has been no progress to improve the disappointing situation in the routine analysis of salivary cortisol. With regard to the results of this study, which especially focused on stress research, we intend to call attention to this problem and would like to emphasize that standardization of salivary cortisol measurement should urgently be improved to the common level of clinical chemistry, particularly by implementation of reference materials, a reference method, comprehensive regular proficiency testing schemes, and adequate commercially available quality control materials. This would provide a reliable and non-invasive method for the determination of cortisol in clinical investigations and stress research.

#### **Conflict of interest statement**

**Authors' conflict of interest disclosure:** The authors stated that there are no conflicts of interest regarding the publication of this article.

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