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Salivary cortisol measurement in horses: immunoassay or LC-MS/MS?

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ABSTRACT

The aim of the present study was to measure salivary cortisol concentrations of horses before and after hypothalamic-pituitary-adrenal (HPA) axis stimulation by means of liquid chromatography-tandem-mass spectrometry (LC-MS/MS) and an immunoassay (cELISA) for method comparison. Nine clinically healthy horses participated in the study. An ACTH stimulation test was performed. Saliva samples were collected before (T0) and 60 (T60) min after intravenous injection of 1 μg/kg BW synthetic ACTH1-24. LC-MS/MS was assessed for the determination of equine salivary cortisol. The results of these measurements were then compared to the results obtained by a cELISA, which has previously been validated for use in horses. The Pearson correlation coefficient was calculated and showed no correlation at T0 ($r = -0.2452$; $P = 0.5249$) and significantly correlated results at T60 ($r = 0.8334$; $P = 0.0053$). Bland-Altman-Plots of T60 revealed that immunoassay measurements led to higher outcome values than LC-MS/MS. On average, immunoassay results were 2.3 times higher. Poor agreement between both methods at T0 is potentially a consequence of inaccuracy in the very low measuring range of the immunoassay, and to a smaller extent, structurally similar cross-reacting agents and matrix effects, which might bias the results. Overestimation of immunoassay results at T60 might be due to different standardization of both methods, non-avoidable matrix effects on the antigen-antibody interaction in the ELISA, and possibly cross-reactions of other steroids. While immunoassay measurements of equine salivary cortisol yielded higher but reasonably correlated results for elevated cortisol concentrations after stimulation of the HPA axis, LC-MS/MS provided more accurate results, particularly for baseline cortisol concentrations close to the limit of detection of the ELISA.

1. Introduction

For more than 2 decades, the measurement of salivary cortisol has been utilized for the assessment of the hypothalamic-pituitary-adrenal (HPA) axis function in horses [1]. It reflects the free, biologically active form of serum cortisol. In equine stress research, saliva sampling offers a simple and non-invasive method to assess the HPA axis in horses that are used to be handled by people. Salivary cortisol measurements have been used to investigate stress in horses during isolation [2], novel manipulations [3], weaning [4], transportation [5–8], hot iron branding and microchip implantation [9], training and competition [10–14], or during work in the presence of an audience [15]. Salivary cortisol has also been measured after HPA axis stimulation by means of an ACTH stimulation test. Stimulated salivary

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cortisol values may be a more robust measure of stress, since basal cortisol appears to be highly sensitive to confounding stimuli, such as seasonal variability or the diurnal rhythm within cortisol concentrations [16–18]. Consequently, ACTH stimulation tests have been used in several studies for the investigation of horses with a gastric disease [19,20], stereotypes [21], or overtraining [22].

All of the above-mentioned studies used immunoassays for salivary cortisol measurements. However, immunoassay accuracy and precision may be reduced due to interacting effects, such as matrix effects of sample constituents on the antigen-antibody interaction, potentially also cross-reactivity of related steroid metabolites, for example, cortisone, or restricted analytical range [23]. First applied in 2003 [24], liquid chromatography-tandem-mass spectrometry (LC-MS/MS) has become the gold standard method for salivary cortisol measurements in humans [23,25,26]. It does not suffer from the cross-reactivity issues commonly observed with antibody-based assays of steroids and permits simultaneous measurement of several steroids [27]. In horses, LC-MS/MS has been used for the determination of steroid profiles in serum and urine [28,29], as well as in plasma after an ACTH stimulation test [30]. To the best of our knowledge, however, no study has so far analyzed equine salivary cortisol using LC-MS/MS. The aim of the present study was, therefore, to measure salivary cortisol of horses by means of LC-MS/MS and an immunoassay for method comparison.

2. Materials and methods

2.1. Sample population

The study was approved by the cantonal authority for animal experimentation, the Veterinary Office of the Canton of Bern (Switzerland) (License number 27608/BE26/16). Owners of the participating horses gave their written consent. Nine horses were included in the analysis.

2.2. Experimental setup

Each horse underwent an ACTH stimulation test. The tests were performed in the horses' normal environment. They were not exercised for 2 h before the test and did not take part in competitions, nor were they transported in the preceding 48 h, in order to avoid potentially confounding HPA axis stimulation. They received no medication except for over-the-counter nutritional supplements. The weight of the horses was estimated with the following formula: $(\mathrm{kg}) = (heartgirth^2 \times body\ length)/(11{,}880\ cm^3)$ [31]. Only horses in good health status, assessed in a clinical examination, were included in the study. During the test, the horses stayed in their box or open stable. Water buckets were removed and self-watering troughs covered with plastic bags so that horses could not drink during the test to avoid dilution of saliva.

2.3. ACTH stimulation test

For the ACTH stimulation test, each horse received an intravenous injection of 1 μg/kg BW synthetic ACTH1-24 (Synacthen tetracosactidum 0.25 mg/mL equivalent to 25 IU/mL; Novartis, Vilvoorde, Belgium). The dosage was in accordance with previous publications [19,20,32,33]. Saliva samples were collected using "Salivette for cortisol" (Sarstedt, Nuembrecht, Germany) before (minute 0–T0) and after (minute 60–T60) ACTH administration. The Salivette swab was placed in the mouth of the horse until it was completely soaked with saliva. The swab was then placed into the tube and stored in a box with cool packs. After return to the laboratory, the Salivettes were directly centrifuged at room temperature for 10 min at 185 × *g* and stored at −20°C until the analysis.

2.4. Steroid analysis by LC-MS/MS

Saliva samples were centrifuged at 5,500 × *g* for 15 min. 500 μL of the supernatant were then diluted with 500 μL water and isotopically labeled D4-cortisol, and D8-cortisone (Sigma-Aldrich, Switzerland) added to a final concentration of 1.8 ng/mL each. Samples were vortexed and then purified using solid-phase extraction on an OasisPrime HLB 96-Well Plate (Waters, UK). LC-MS/MS measurements were carried out by coupling of a Vanquish UHPLC to a QExactive Orbitrap Plus (both Thermo Fisher Scientific, Switzerland). The separation was achieved using an Acquity UPLC HSS T3 Column, 100Å, 1.8 μm, 1 mm × 100 mm (Waters, UK). Mobile phases A and B consisted of water +0.1% formic acid and methanol +0.1% formic acid, respectively (all UHPLC grade; Sigma-Aldrich, Switzerland). Analytes were eluted using a linear gradient from 46% to 73% B over 8 min. The mass spectrometer was operated in positive ion mode using an electrospray ionization source.

Calibration was performed using an 8-point calibration curve ranging from 0.01 to 300 ng/mL prepared in synthetic saliva based on the recipe by Shellis [34,35]. Accuracy was assessed by independent measurements of the same equine sample (n = 3), precision by standard addition to equine samples (3 independent measurements, 3 levels of the added standard each). Reproducibility was assessed using quality control samples prepared at 4 different levels in synthetic saliva (0.3 ng/mL, 3 ng/mL, 7 ng/mL, 100 ng/mL; 3 independent experiments at each level). Data analysis was performed using TraceFinder 4.0 (Thermo Fisher Scientific, Switzerland).

2.5. Cortisol analysis by immunoassay

A saliva-specific competitive enzyme immunoassay (cELISA, Salimetrics, Newmarket, United Kingdom) was used for cortisol analysis. The manufacturer states a functional sensitivity of 0.28 ng/mL and cross-reactivity for 14 endogenous and synthetic steroids is reported to be <1% each (19.2% for dexamethasone) [36]. The assay had been used according to our previous validation for use in horses [33]. Intra-assay and inter-assay coefficients of variations were 6.4% and 4.0%, respectively.

2.6. Statistical analysis

All statistical analyses were performed using R studio Team, 2016 [dataset] [37]. Data of all 9 horses were tested

for normality of distribution via the Shapiro Wilk test and visual inspection (Histograms and QQplots). Correlations between cortisol values measured via LC-MS/MS and immunoassay were investigated by means of Pearson correlation coefficient (function cor.test). The comparison was visualized (R base package and ggplot2 package) using a scatterplot, including linear regression lines (Fig. 1), as well as the combined box- and line plots (Fig. 2). Bland-Altman plots and modified Bland-Altman plots (Fig. 3) were performed to assess agreement between the absolute value of paired salivary cortisol results from LC-MS/MS and immunoassay, whereas the modified version incorporates LC-MS/MS as the gold standard [38]. Just like the original Bland-Altman plot, the modified form displays the difference between LC-MS/MS and immunoassay values on the y-axis (in percent) but plots them against LC-MS/MS salivary cortisol values on the x-axis instead of using the average of LC-MS/MS and immunoassay values [39]. Good agreement was specified as a mean difference near zero and 95% or more of the data falling within 1.96 × standard deviations of the mean difference. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Sample population

Data are reported as mean ± SD (range). The age of the 9 horses included in the analysis was 10.4 ± 2.9 (8 to 17) yr. Four of them were mares and 5 geldings. Breeds included Warmbloods (n = 3), Thoroughbreds (n = 4) and Franches-Montagnes (n = 2).

3.2. Validation of the LC-MS/MS method for equine samples

Measurement of salivary cortisol and cortisone concentrations has been validated for routine clinical use in human samples according to FDA guidelines [40] with respect to accuracy, precision, reproducibility, sensitivity, recovery, and stability. By using the same method, accuracy, precision, reproducibility, and sensitivity were determined for equine samples. The results of this validation are summarized in Table 1; all validation parameters were well within limits. The method shows a large linear range over 4 orders of magnitude, and performance is comparable to application in human samples [41].

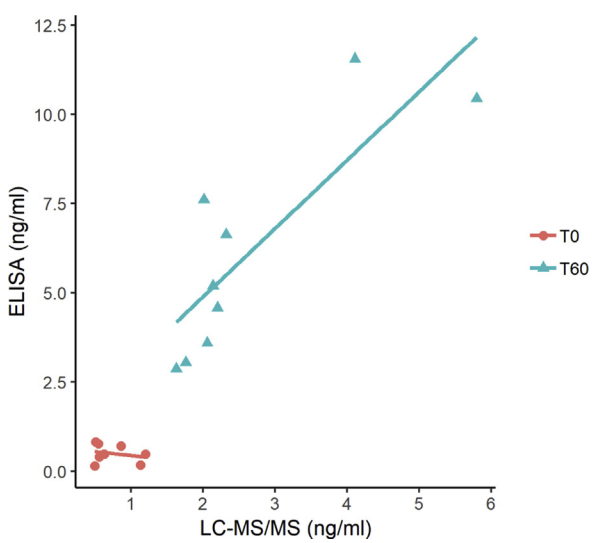

Fig. 1. Scatterplot, including linear regression lines that demonstrate the correlation between cortisol values (ng/mL) measured via ELISA and LC-MS/MS before (0 min–T0) and after (60 min–T60) ACTH administration.

3.3. Comparison of cortisol measurements by ELISA and LC-MS/MS

Statistical analysis revealed a non-normal distribution of cortisol values at T0 and T60. Prestimulatory cortisol values (T0) that were lower than 1.8 ng/mL did not correlate between the 2 methods ($r = -0.2452$; $P = 0.5249$). However, correlation analysis of higher cortisol concentrations (between 1.8 and 11.6 ng/mL), seen after stimulation (T60) showed significantly correlated results ($r = 0.8334$; $P = 0.0053$). Visualization via box- and line plots (Fig. 2) and Bland-Altman-Plots (Fig. 3) of T60 show that immunoassay measurements lead to higher results in comparison to LC-MS/MS measurements. At T0, the immunoassay shows only a small bias (10.6%), which increases to over 50% at T60. The confidence intervals are comparable in both cases (±30%). Overall, mean immunoassay values were 2.3 times higher than LC-MS/MS values.

4. Discussion

To the best of our knowledge, this is the first study reporting salivary cortisol measurements by means of LC-MS/MS in horses and comparing the results from this accepted gold standard method in human research to those obtained by an immunoassay. We found a high discrepancy between baseline cortisol measurements obtained by the two methods. In addition, salivary cortisol values after ACTH stimulation revealed constantly higher values using the immunoassay in comparison to LC-MS/MS.

Our validation of LC-MS/MS did not show any statistically significant difference between species (human and horse), and all tested parameters (accuracy, precision, reproducibility, sensitivity, recovery, and stability) were well within limits. The large linear dynamic range indicates no dilutional effects. Although matrix effects can affect LC-MS/MS, the use of isotopically labeled cortisol as an internal standard largely circumvents this problem. Of course, as there are slight differences in the LC retention time of the internal standard compared to the analyte (< 3 s), both the internal standard and analyte might be affected by ion suppression to a slightly different degree. A source of error might also be the use of a synthetic saliva formulation, which potentially differs somewhat from the composition of natural saliva, leading to unexpected matrix effects, which could have a negative impact on quantitation.

Poor agreement between LC-MS/MS and immunoassays for cortisol concentrations below 1.8 ng/mL have been reported in human samples [25]. The results of the present study confirm this finding in horses. Baseline cortisol concentrations of the current sample population measured

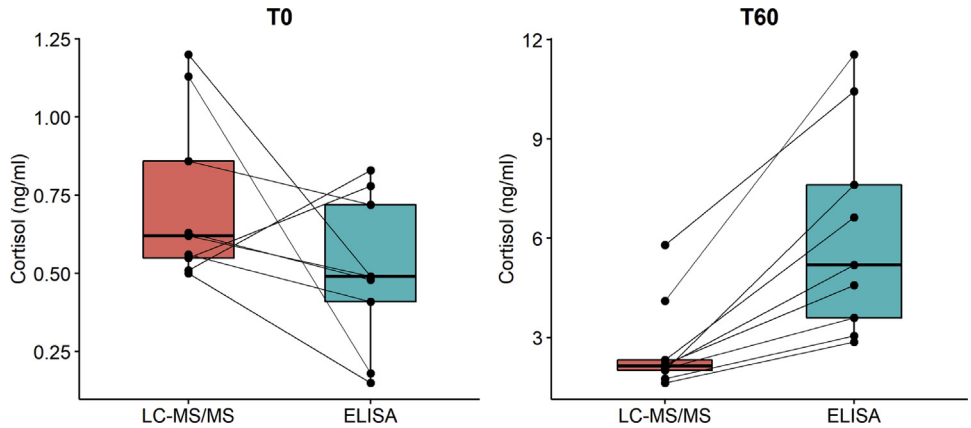

Fig. 2. Combined box- and line plots comparing cortisol measurements between 2 methods–ELISA and LC-MS/MS before (0 min–T0) and after (60 min–T60) ACTH administration.

with the immunoassay were between 0.15 and 0.83 ng/mL and stimulated values between 2.87 and 11.55 ng/mL. Thus, baseline values were close to the limit of detection of the immunoassay (0.28 ng/mL functional sensitivity). As the sigmoidal calibration curve of the assay is flat in the low concentration range, inaccuracy is more likely than in concentration ranges where the calibration curve is linear. A further possible explanation is a non-linear behavior for

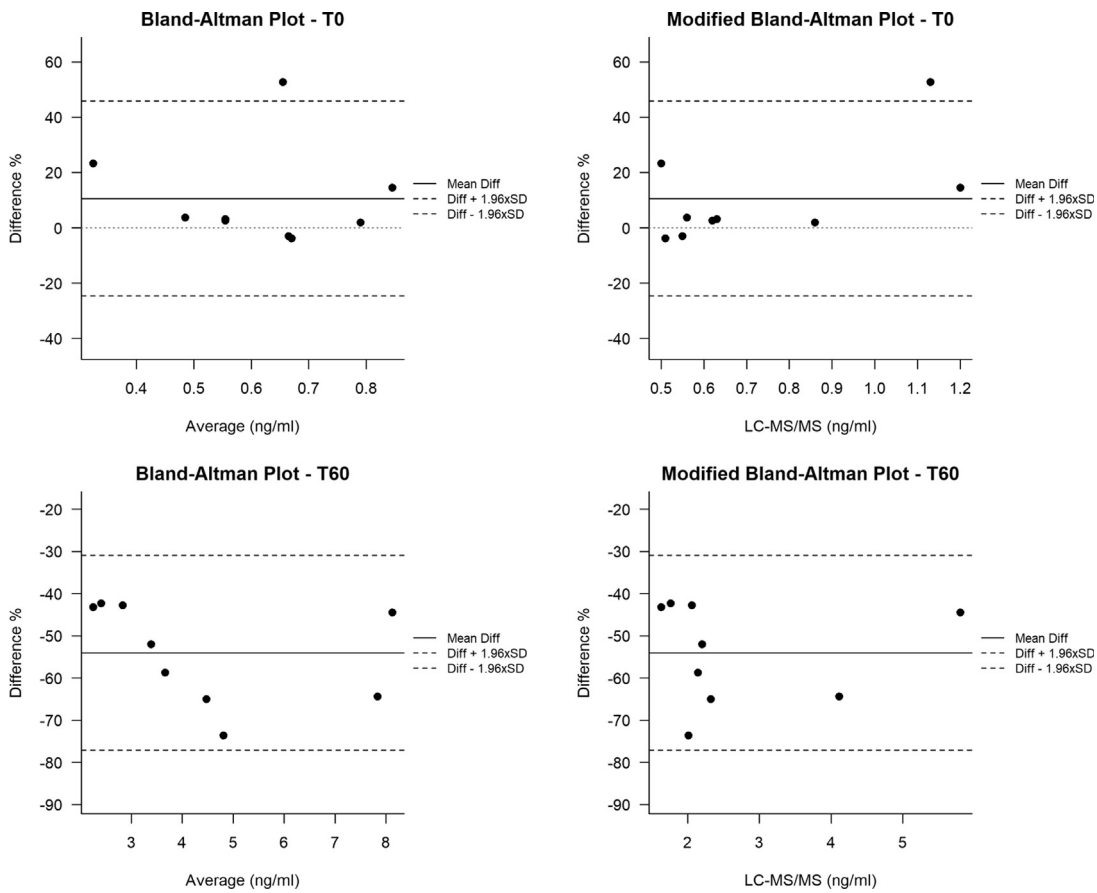

Fig. 3. Bland-Altman-Plots describing the degree of agreement between the 2 salivary cortisol measuring techniques–ELISA and LC-MS/MS–before (0 min–T0) and after (60 min–T60) an ACTH challenge test. The 2 plots on the left side display the percentage difference between the 2 methods (y-axis) against the mean (x-axis). The 2 plots on the right side show modified Bland-Altman-Plots presenting the percentage difference between the 2 methods (y-axis) against LC-MS/MS. The solid line shows the mean difference, whereas the upper dashed line represents the upper limit of agreement (Diff + 1.96 × SD), and the lower dashed line represents the lower limit of agreement (Diff − 1.96 × SD).

Table 1
Results of the validation of liquid chromatography-tandem-mass spectrometry (LC-MS/MS) cortisol method for equine samples. All validation values were within limits according to FDA guidelines.

Parameter	Result
Accuracy	3.6% RSD
Precision	1.5% RSE
Reproducibility	<15% RSE
Limit of Detection	0.02 ng/mL
Lower Limit of Quantification	0.08 ng/mL
Upper Limit of Quantification	300 ng/mL
Linearity	$R^2 = 0.995$

antibody-binding of cortisol at low levels, especially in the presence of structurally similar cross-reacting interferents, such as cortisone [25,26]. The cortisone cross-reactivity of 0.13%, as declared by the manufacturer of the ELISA-kit that was used in the present study, however, is lower than the cross-reactivity of 4.5% of the immunoassay that was shown to influence low cortisol values in a previous study [25]. Consequently, this effect is important to be kept in mind as a possible biasing factor [27]; however, considering the low cross-reactivity, relevance for the present study appears to be questionable. The potential vulnerability of the assay to salivary proteins, such as alpha-amylase or other matrix components of the saliva has been discussed before [25], and might further exacerbate the reduced precision. Decreased accuracy of salivary cortisol immunoassay measurements is especially important since equine salivary baseline cortisol values are consistently reported to be lower than 1.8 ng/mL [32,33,42], as mentioned above for the current results, and are frequently measured for the evaluation of stress in horses [43]. To conclude, our results indicate that equine baseline cortisol values obtained by immunoassays need to be interpreted with caution and that future studies must take into consideration that salivary cortisol immunoassay measurements in lower ranges might be accompanied by low accuracy.

In the present study, consistently higher cortisol levels were observed for cortisol values measured after ACTH stimulation using the immunoassay, an effect that has equally been demonstrated in human saliva samples [25,26,39]. According to Bae et al [25], this discrepancy is primarily due to differences in the standardization of the 2 methods. Examination of the immunoassay calibrators in the study of Bae et al [25] revealed that the setpoints were 2.37-fold higher than the cortisol concentrations measured by LC-MS/MS. The observed difference was similar to the offset found in clinical samples and is also similar to the 2.3-fold difference in our samples. Note that we did not verify the immunoassay calibrators in this study, and can, therefore, only speculate about the reason for this offset. If the concentration of the setpoints was the sole problem, it could be solved by standardizing immunoassays in reference to LC-MS/MS as it would merely be a scaling problem. There is, however, evidence for other contributing factors, such as the aforementioned cross-reactivity [27] or matrix effects. While pronounced cross-reactivity seems unlikely in the present study, other authors found it to be relevant in their investigations [24,26,44,45]. The reason why LC-MS/MS assays do not suffer from such problems is the fundamentally different measurement principle. Immunoassays rely on interactions between antigens and antibodies, and are, therefore, prone to errors for structurally extremely similar molecules, such as steroids. LC-MS/MS analysis, on the other hand, is based on the physicochemical properties of the analyte, such as the molecular formula, chemical structure, and hydrophobicity. Compounds that have been reported to show strong cross-reactivity in immunoassays, such as 6β-hydroxycortisol, 11-deoxycortisol, 21-deoxycortisol, and prednisolone, can easily be differentiated by LC-MS/MS from cortisol as they are both separated by chromatography (differences in hydrophobicity) and mass spectrometry (differences in the molecular formula). Nonetheless, LC-MS/MS can also suffer from matrix effects. Fortunately, these can be efficiently dealt with by the use of isotopically labeled internal standards as employed in our study.

Limitations of the present study are the small sample size and testing of only one immunoassay, as the transferability of our results to other immunoassays is restricted. The ELISA used is one of several immunoassays that have been utilized for salivary cortisol determination in horses [19–21,33,46]. Furthermore, no formal comparison between LC-MS/MS and the ELISA-kit of low concentration spiked samples was performed to demonstrate the superiority of one method to the other. However, the assumption of LC-MS/MS as the gold standard seems admissible, as our results agree with the findings of previous human clinical research [25,26,39].

In conclusion, equine salivary cortisol measurements with the ELISA-kit that we used may perform satisfactorily for the analysis of elevated concentrations (>1.8 ng/mL), such as after HPA axis stimulation tests. In the future, other immunoassays should be specifically assessed against LC-MS/MS to allow valid conclusions about their research utility. If low baseline concentrations need to be determined, however, LC-MS/MS seems more accurate. Additionally, LC-MS/MS offers the possibility to measure additional steroids in parallel.

CRediT authorship contribution statement

F.J. Sauer: Conceptualization, Software, Formal analysis, Investigation, Writing - original draft, Visualization. **V. Gerber:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **S. Frei:** Investigation. **R.M. Bruckmaier:** Validation, Resources, Writing - review & editing. **M. Groessl:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision.

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The authors declare no conflicts of interest.

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