



The Saliva Cortisol and Amylase Levels Related with Stress Response Compared by Different Analytical Methods

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Abstract

Sampling of salivary cortisol and amylase is a non-invasive method and important for the evaluation of the hypothalamic–pituitary–adrenal axis function and stress levels. This study aimed to compare the values of the salivary cortisol and amylase levels which were measured by three different analytical methods to discuss the alterations of stress levels of samples. The saliva samples of young adults ($n=23$) were collected between 08.00 and 09.00 a.m., noon at 12.00 (before exam) and between 14.00 and 15.00 p.m. (after unaware exam). The samples were measured within the first 48 h, and no freezing/thawing was done. Salivary cortisol and amylase levels of subjects were measured by three different analytical methods as ELISA, chemiluminescence and biosensor methods. Comparison of ELISA and biosensor methods in order to determine the salivary cortisol levels showed a good correlation $y=2.971+0.748x$ ($R^2=0.839$). Salivary amylase concentrations were only detected by ELISA method. Biosensor can be offered as an alternative analytic method to the conventional determination method ELISA. It can be preferred because of the detection/information effectiveness, low cost, fast results and specificity characteristics.

Highlights

- Saliva is a non-invasive alternative body fluid which will represent health status as blood samples; however, method comparisons are necessary to speculate on the accuracy of worked biochemical parameters.
- Biosensor methods could be a point of care tests, easy and cheap, related with the logic of biosensor methods, and strip tests for saliva might be developed.
- The relevancy of saliva samples are increasing among routine biochemical parameters.
- Saliva can work as a diagnostic tool.

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Introduction

Saliva samples can be used as a non-invasive monitoring tool for stress analysis by measuring the cortisol levels which are one of the biomarkers of stress in addition to salivary enzymes, α -amylase and glucocorticoids [1–3]. Recently, saliva samples have been preferred instead of blood samples as the salivary cortisol levels correlate to the cortisol levels in serum samples. Since unbound cortisol is biologically active, it is more useful to determine the cortisol level in saliva [4–6].

Saliva cortisol, measured by traditional methods, is in free form and delivered directly to the target tissues along the capillaries. The cortisol level of saliva reliably reflects hypothalamic–pituitary–adrenal (HPA) axis activity and has been used in many studies as a biological stress marker [7].

Saliva α -amylase enzyme, which is produced by the salivary glands, was released as a result of activation of the autonomic nervous system [8]. Both physiological and psychological stressors are used to demonstrate the relationship between saliva α -amylase enzyme and stress. However, despite the existence of many studies explaining this relationship, contradictory results are also encountered [9]. Saliva α -amylase enzyme can be used as an indicator in non-invasive measurement of stress, and these measurements generally use the substrate method (CNP3) [10].

Enzyme-linked immunosorbent assay or autoanalyser were used to detect salivary cortisol and amylase levels in many studies. However, immunoassay accuracy and precision may be reduced due to interacting effects, such as matrix effects of sample constituents on the antigen–antibody interaction. Also, potentially restricted analytical range can be observed [11, 12]. Successful measurement of cortisol (steroids) depends on a very sensitive and specific analysis method. Analysis of steroids by biosensor has previously been shown to be superior to immunoassay, in terms of both increased specificity and sensitivity [13, 14].

Alfa-amylase and cortisol levels can be measured by different methods, but no studies comparing them with each other have been found. Therefore, in this study, α -amylase enzyme activity and cortisol levels were measured according to three different methods, and the methods were evaluated in terms of cost, time and feasibility. In addition, data on these methods was evaluated in terms of their relationship with stress in young adult individuals.

Materials and Methods

Collection of Saliva Samples

Saliva samples were collected using an oral swab (SARSTEDT brand Salivette oral swab) made of polypropylene/low-density polyethylene (LD-PE) material designed specifically for cortisol. These tubes provide high analytical results from small quantities of samples.

In order to keep the standardization related with circadian rhythms, saliva samples were collected between 08.00 and 09.00 a.m. Nevertheless, it was convened at 12.00 (pre-stress) and between 14.00 and 15.00 p.m. (post-stress/after an unaware exam hour) to evaluate

its relationship with stress. Within 60 min before the test, individuals were not allowed to smoke, eat, drink liquids or brush their teeth. They were asked to soak the swab with saliva for 1 or 2 min until they are saturated completely. Tubes were waited 30 min at room temperature and then centrifuged for 10–15 min at approximately 3200 g. After centrifugation, the swab and small insert were thrown away, and the large outer tube was stored at -80°C until analysis.

The 23 healthy young adult of the Faculty of Dentistry students were recruited to the study. Exclusion criteria were presence of any disease that can affect hypothalamic–pituitary–adrenal (HPA) axis.

Analysis Methods

We divided saliva samples into three sets: the first set was for ELISA kit, second set was for biosensor, and the other set was for chemiluminescence immunoassay.

ELISA

Salivary cortisol and salivary amylase were measured by using a high-sensitivity ELISA kit (Diametra-Immunodiagnostic System; Lot:4817A; USCN SEB482Hu Enzyme-Linked Immunoabsorbent Assay Kit, for Amylase Alpha 1, Salivary (AMY1) Lot:L181030297), according to the manufacturer's instructions, respectively.

Biosensor

In the current study, a previously optimized method is used in the biosensor prepared for cortisol and amylase.

Sensor Fabrication

The cortisol and amylase sensors have a three-electrode system consisting of a nanopolymer-modified gold working electrode, reference electrode and counter electrode. The bioactive layer of the polyaniline nanopolymer-modified gold working electrode has also anti-cortisol and anti-amylase antibody on its surface, because the biosensor system's working principle is depending on a specific interaction between antigen and antibody of cortisol and amylase. When antigen–antibody interaction occurs, potential differentiation detection with bioelectrochemical methods happens.

Differential pulse spectroscopy and cyclic voltammetry were used to characterize the electrical performance of the fabricated sensor system and identify the electrical nature of molecules used in each step of the immunoassay.

Polyaniline conducting nanopolymer (Sigma, USA) was used as a bioactive layer component for surface expansion. The electrodes were immersed in aniline solution for electrochemical polymerisation. Whereas a capture antibody specific to cortisol was used for the detection of cortisol in buffer solutions which were the immunoassay reagents of the salivary cortisol ELISA kit, a capture antibody specific to amylase was used for the detection of amylase in buffer solutions which were the immunoassay reagents of the salivary amylase ELISA kit.

Electrochemical differential pulse spectroscopy has been used for characterization of binding events on the surface of sensors of working electrodes. In our method, we used an electrochemical differential pulse spectroscopy to characterize the binding of the different entities on the electrode surface as they create a Coulombic potential, which could be screened as differential pulse changes. Electrical double-layer capacitance is reflective of the coulomb potentials developed due to biomolecular binding. Experimental parameters used in this study included $V_{rms}=10$ mV and frequency range of 10 Hz to 10 kHz [15, 16].

Calibration of Sensor Platform for Detection

Prior to testing the human salivary cortisol and salivary amylase samples, the sensor was first calibrated by calibration standards in the PBS buffer. The target antigen was calibrated for signal changes occurring due to binding cortisol and amylase antigen with capture antibody probes on the sensing electrodes and binding of detection antibody to antigen–capture antibody conjugate on the sensing electrodes. The calibration response was performed for a total of $n=10$ replicates, and errors were calculated as the SD over mean. Curve fitting was performed to establish a correlation between varying concentrations of the calibration standards with respect to differential pulse measured. As a negative control, each of the sensor arms was also tested for non-specific signal with the blank buffer solution and cortisol and amylase from healthy volunteers.

Detection of Human Salivary Cortisol and Salivary Amylase Samples

The sensor platform was tested for feasibility and accuracy of detection of cortisol and amylase at human salivary to show proof of feasibility for detection using differential pulse spectroscopy toward application in companion diagnostics. A total of 23 samples were tested, and a semi quantitative analysis was used to compare with results obtained from ELISA. The samples were not diluted because of the wide detection range of the designed sensor. The samples were added at a volume of 20 μ L to the sensor's working cell. The sample was incubated for 1 min. Differential pulse measurements were performed without the need for any wash steps.

Chemiluminescence Immunoassay

The other set of saliva samples were used to measure cortisol and amylase levels in saliva using cortisol and amylase testing as in serum and plasma samples. Saliva samples were transferred to the sample cups and analysed by the Beckman Coulter DxL 800 autoanalyser.

Amylase measurement is carried out enzymatically that utilizes 2-chloro-4-nitrophenyl- α -D-maltotrioxide (CNPG3) as substrate [17]. This substrate reacts directly with α -amylase and does not require the presence of ancillary enzymes. The release of 2-chloro-4-nitrophenol (CNP) from the substrate and the resulting absorbance increase per minute are directly related to the α -amylase activity in the sample. The resulting increase in absorbance can be measured by spectrophotometrically at 410/480 nm.

Cortisol assay is a competitive binding immunoenzymatic assay. A sample is added to a reaction vessel with rabbit antibody to cortisol, cortisol-alkaline phosphatase conjugate and paramagnetic particles coated with goat anti-rabbit capture antibody. Cortisol in the sample competes with the cortisol-alkaline phosphatase conjugate for binding sites on a limited amount of specific anti-cortisol antibody. Resulting antigen: antibody complexes bind to the capture antibody on the solid phase [18, 19].

Statistical Analysis

Statistical analysis was performed using SPSS 18 statistics program, Jamovi and Analyse-it programs. The means and standard deviations of the samples were taken in all applied methods. To determine whether the distribution of data was normal, the Shapiro–Wilk test was used. Since the data were distributed homogeneously, statistical analysis was done by parametric tests. The results are presented as mean and standard deviation levels. Comparisons between the groups of data were evaluated by use of parametric one-way ANOVA and the Tukey post hoc tests. Spearman's correlation coefficient was calculated to assess the strength and direction of the compared data. The results were evaluated with a confidence interval of 95% and statistical significance was set at a *p*-value of less than 0.05. Linear regression was carried out Passing-Bablok regression. Regression analysis of enzyme-linked immunoabsorbent assay (ELISA), chemiluminescence immunoassay (autoanalyser) and biosensor data obtained in the method comparison study was performed. Differences between methods were evaluated by calculating bias and limits of agreement using Bland–Altman charts.

Results and Discussion

Many researchers working in different fields use saliva samples to measure stress parameters. However, saliva sampling and detecting methods need optimizations when evaluating stress-related parameters. The most commonly used saliva stress markers are cortisol and amylase.

In the current study, a biosensor method was used to determine the amounts of cortisol and α -amylase in saliva samples. The levels of compatibility and comparison of other measurement methods used as a reference to the new measurement method were examined by statistical analysis. In a second step, it was discussed whether there is a difference in saliva cortisol and amylase levels during stress status.

In 23 healthy young adults (17 female and 6 male), the mean age was 20.5 ± 0.95 years (range: 20–24 years). The mean age of female and male participants of groups was 20.2 ± 0.43 and 21.17 ± 1.60 ($p=0.001$) respectively. The mean and standard deviations of cortisol and amylase concentrations obtained in salivary samples drawn in the morning, pre-stress and post-stress are shown in Table 1.

The results of all three methods in our study suggested that there were differences in cortisol concentrations of saliva samples during the morning hours, which were high, decreased at pre-stress and increased at post stress. However salivary amylase concentration was decreased in the morning and increased before and after stress.

The performance of the methods used to determine the levels of cortisol and α -amylase was examined at three different time periods as in the morning, pre-stress and

Table 1 The salivary cortisol (SC) and amylase (sAA) levels determined by different three analytical methods at different three time intervals

Methods	Time of sampling			<i>p</i> -value
	Morning (08.00–09.00 a.m.) Mean ± SD	Pre-stress (12.00) Mean ± SD	Post-stress (14.00–15.00 p.m.) Mean ± SD	
SC-ELISA (µg/dL)	10.76 ± 3.30	8.64 ± 3.33	12.67 ± 5.96	0.014
SC-biosensor (µg/dL)	12.12 ± 7.23	9.47 ± 3.65	12.63 ± 3.61	< 0.001
SC-autoanalyser (µg/dL)	9.66 ± 2.93	5.76 ± 2.54	7.13 ± 2.06	0.019
sAA-ELISA (ng/mL)	17.92 ± 4.21	30.65 ± 9.74	117.52 ± 38.1	< 0.0001

SC salivary cortisol, sAA salivary α-amylase, SD standard deviation

post-stress in young adult individuals. In Fig. 1, the findings of ELISA, biosensor and auto-analyser method results are presented for the young adult individuals.

Saliva cortisol measurement results obtained by three methods were evaluated statistically. Regression analysis was carried out to examine the relationship between them,

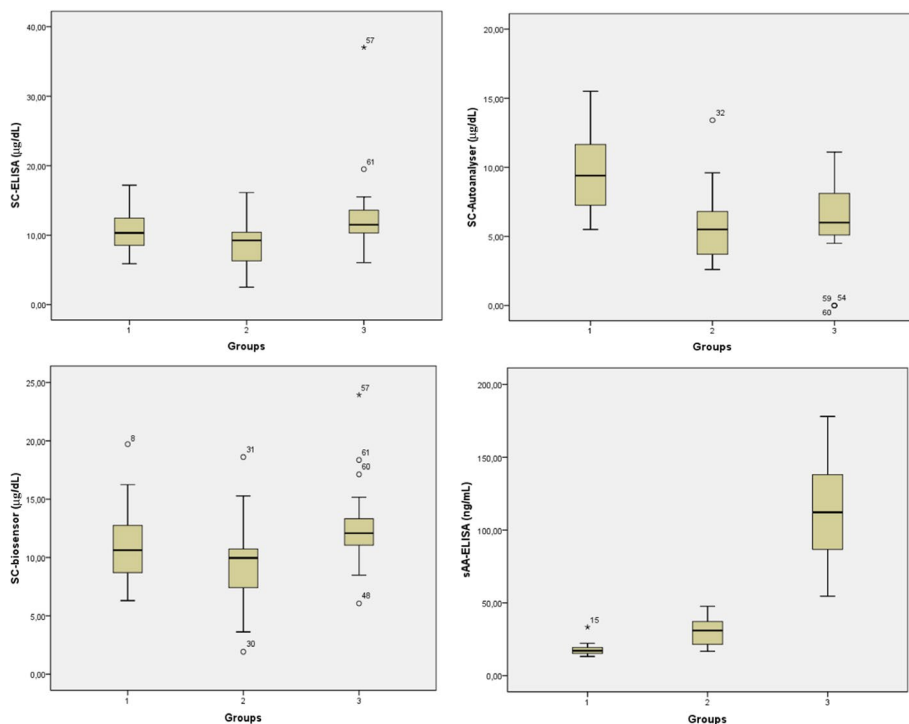
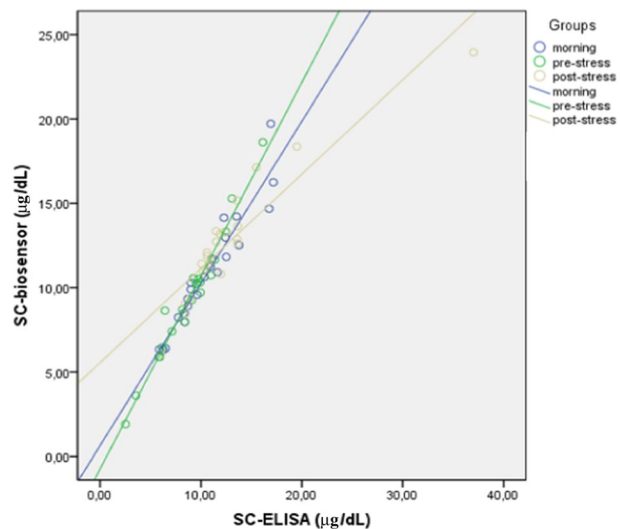


Fig. 1 Performance of methods used to determine cortisol and amylase levels in samples taken at different times. Group 1: 08.00–09.00 a.m. (morning); group 2: before exam (pre-stress); group 3: after unaware exam (post-stress)

Table 2 Post hoc analysis for the comparative analysis of different analytical methods of measurement for cortisol levels between study groups

Methods of measurement for salivary cortisol ($\mu\text{g/dL}$)	Study groups	Morning	Pre-stress	Post-stress
ELISA	Morning (08.00-09.00 a.m.)	–	0.235	0.308
	Pre-stress (12.00)	0.235	–	0.007*
	Post-stress (14.00-15.00 p.m.)	0.308	0.007*	–
Autoanalyser	Morning (08.00-09.00 a.m.)	–	0.0009*	0.0001*
	Pre-stress (12.00)	0.0009*	–	0.996
	Post-stress (14.00-15.00 p.m.)	0.0001*	0.996	–
Biosensor	Morning (08.00-09.00 a.m.)	–	0.329	0.260
	Pre-stress (12.00)	0.329	–	0.011*
	Post-stress (14.00-15.00 p.m.)	0.260	0.011*	–

*The statistical significance is marked with asterisks $p < 0.05$ *

Fig. 2 Correlation of SC-ELISA and SC-Biosensor methods

and this relationship was expressed as the correlation coefficient. The values obtained from all three methods were evaluated by Spearman correlation analysis, and a significant difference was found between the three methods.

Each method reveals salivary cortisol concentrations close to each other for all three times intervals; salivary amylase concentrations were detected only in the ELISA method (Table 1). Besides, by ELISA and biosensor method, post-stress salivary cortisol levels were found to be higher than pre-stress. Cortisol levels of saliva samples were found to be higher in the morning than pre- and post-stress period measured by autoanalyser method (Table 1). Details of post hoc analysis for comparative analysis of different methods between different study groups are given in Table 2.

When we pooled all the salivary samples of the subjects for three methods, 69 samples were obtained for each method. There were significant positive correlations between ELISA and biosensor levels ($r=0.965^{**}$; $p=0.0005$) (Fig. 2). The evaluation of salivary cortisol levels indicated that the correlation of cortisol levels measured by

biosensor is in strong agreement to the measurements done by the traditional ELISA method.

On the other hand, cortisol biosensor values appear to be slightly higher than the cortisol ELISA levels. The differences between the methods are, firstly, the biosensor has higher sensitivity than ELISA. Secondly, the current reduction caused by impurities increases the biosensor results because the current is inversely proportional to the solution concentration.

The distribution of cortisol results of ELISA method and autoanalyser method is as shown in Fig. 3. The regression analysis for these two methods are $R^2=0.012$, $a=6.2378$ $b=0.0811$. The equation for these analyses was found as $y=6.238+0.0811x$, which proved that the results of ELISA and autoanalyser method

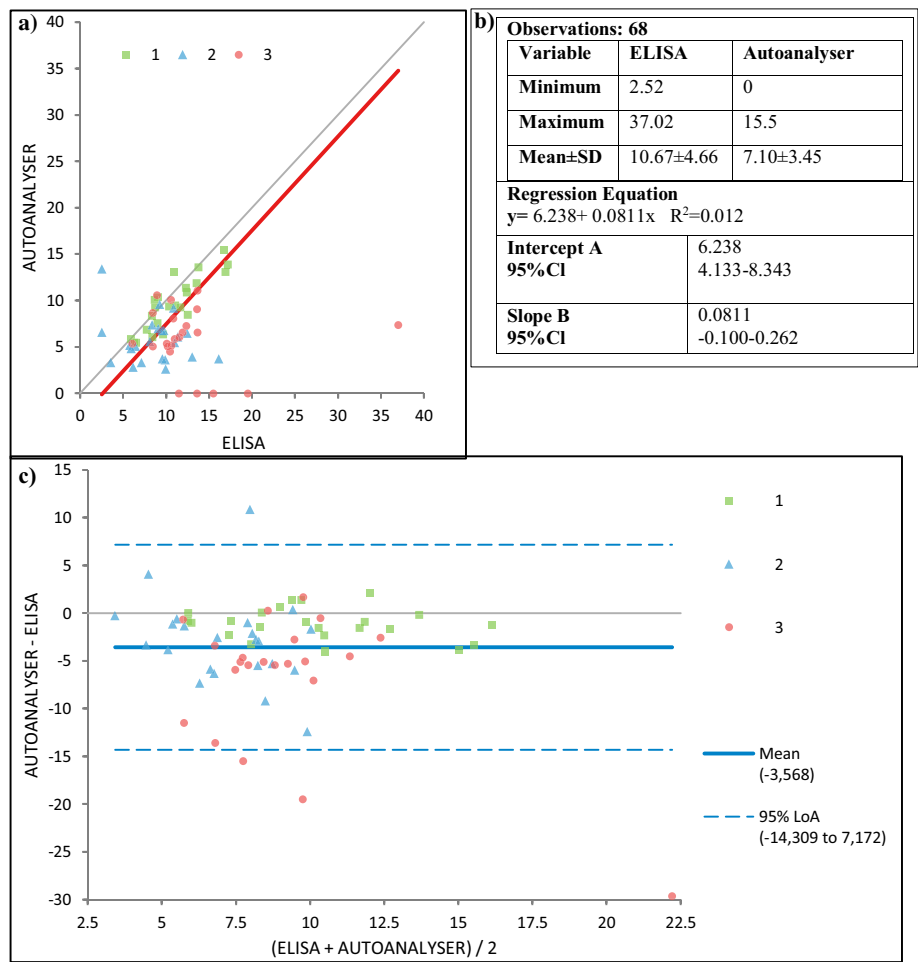


Fig. 3 **a** Distribution of cortisol results between ELISA method and Autoanalyser method. **b** Numerical values of the distribution of cortisol results. **c** Bland–Altman plot of cortisol results between ELISA method and Autoanalyser method. (Green: 08.00–09.00 (morning); blue: before exam (pre-stress); pink: unaware exam (post stress))

were statistically different ($r=0.109$; $p=0.374$). The present data showed that the mean concentration of salivary cortisol measured by autoanalyser was significantly different from that measured by ELISA.

The distribution of cortisol results of ELISA method and biosensor method is as shown in Fig. 4. The result of the regression analysis for these two methods is $R^2=0.839$, $a=2.971$, $b=0.748$. The equation of these analyses was found as $y=2.971+0.748x$, which proved that the results of ELISA and biosensor method were not statistically different ($r=0.916$; $p=0.0007$).

When we compared the biosensor method to other methods, it was determined that the results of the ELISA method were similar to the biosensor, but they were different from the other method, chemiluminescence immunoassay.

The distribution of cortisol results between Autoanalyser method and biosensor method is as shown in Fig. 5. The regression analysis of these two methods is $R^2=0.019$, $a=9.983$, $b=0.153$, and the equation was found as $y=9.983+0.153x$. It was shown that the results

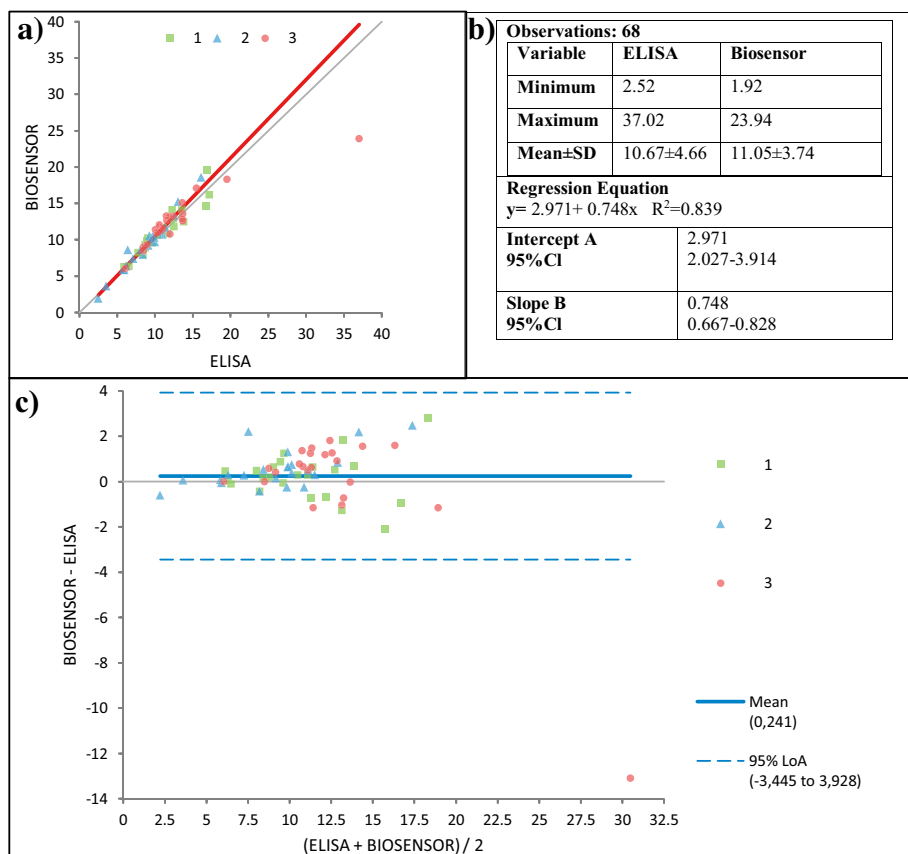


Fig. 4 **a** Distribution of cortisol results between ELISA method and Biosensor method. **b** Numerical values of the distribution of cortisol results. **c** Bland–Altman plot of cortisol results between ELISA method and Biosensor method. (Green: 08.00–09.00 (morning); blue: before exam (pre-stress); pink: unaware exam (post stress))

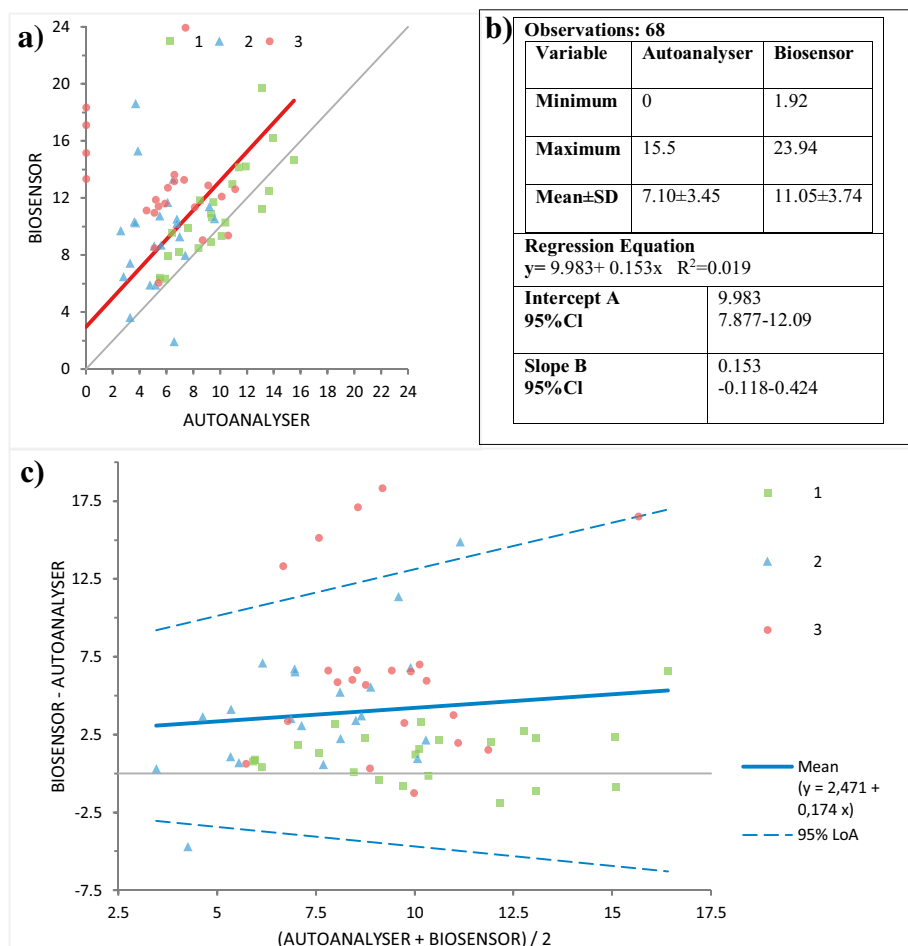


Fig. 5 **a** Distribution of cortisol results between Autoanalyser method and Biosensor method. **b** Numerical values of the distribution of cortisol results. **c** Bland–Altman plot of cortisol results between Autoanalyser method and Biosensor method. (Green: 08.00–09.00 (morning); blue: before exam (pre-stress); pink: unaware exam (post stress))

of the autoanalyser method and biosensor method were statistically different ($r=0.138$; $p=0.264$).

Saliva α -amylase levels could not be determined by autoanalyser and biosensor. The biosensor method developed is not suitable for determining the level of amylase. It was observed that the correct measurement range of the ELISA kit used for amylase was narrow, and there was no reproducibility in the study with the saliva sample. We think that this is due to the lack of method validation for saliva samples, although the kit is compatible with all body fluids.

As a result of the studies conducted, it was seen that the biosensor method developed for cortisol is an alternative method due to low cost, fast result, specificity and high detection/information efficiency compared to ELISA.

Conclusions

Although saliva collection and analysis show some drawbacks, with increasing analysis and technological advances, it has been recognized as an attractive diagnostic fluid for the detection of various saliva biomarkers.

The acceptance of any new methodological approach to assessment of cortisol and α -amylase in a given research environment depends on the ease of saliva sampling, the storage conditions and the reliability of available assays for analysis. Given these characteristics, the cost of the new technique should be advantageous over other conventional methods.

Biosensors, which are the laboratory technology of the twenty-first century, draw intense attention due to their advantages compared to classical methods [15, 16]. In the current study, a new biosensor was designed to determine saliva cortisol amounts. In line with the obtained results, the biosensor method has been demonstrated to be applicable. It is thought that the biosensor method can offer saliva cortisol levels as an alternative analytical method with low cost, fast results, high perception and information validity compared to chemiluminescence immunoassay and ELISA methods.

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Data Availability Applicable.

Declarations

Ethics Approval Our study was performed with the approval of the ethics review board of the Gazi University Faculty of Medicine Ethics Committee, and all the participants gave written informed consent. The number of the ethical approval is 831–12.11.2018.

Informed Consent Written consent was obtained from the participants.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

Peer Review Externally peer-reviewed.

References

1. Corbalan-Tutau, D., Madrid, J. A., Nicolas, F., et al. (2014). Daily profile in two circadian markers “melatonin and cortisol” and associations with metabolic syndrome components. *Physiology & Behavior*, 123, 231–235.
2. Booij, S. H., Bos, E. H., Bouwmans, M. E., van Faassen, M., Kema, I. P., Oldehinkel, A. J., & de Jonge, P. (2015). Cortisol and α -amylase secretion patterns between and within depressed and nondepressed individuals. *PLoS One*, 10(7), e0131002.

3. Petrakova, L., Doering, B. K., Vits, S., Engler, H., Rief, W., Schedlowski, M., & Grigoleit, J. S. (2015). Psychosocial stress increases salivary alpha-amylase activity independently from plasma noradrenaline levels. *PLoS One*, 10(8), e0134561.
4. Hofmann, L. F. (2001). Human saliva as a diagnostic specimen. *Journal of Nutrition*, 131(5), 1621S–S1625.
5. Obayashi, K. (2013). Salivary mental stress proteins. *Clinica Chimica Acta*, 425, 196–201.
6. Nater, U. M., Rohleder, N., Gaab, J., Berger, S., Jud, A., Kirschbaum, C., & Ehlert, U. (2005). Human salivary alpha-amylase reactivity in a psychosocial stress paradigm. *International Journal of Psychophysiology*, 55(3), 333–342.
7. Groer, M., Murphy, R., Bunnell, W., Salomon, K., Van Eepoel, J., Rankin, B., et al. (2010). Salivary measures of stress and immunity in police officers engaged in simulated critical incident scenarios. *Journal of Occupational and Environmental Medicine*, 52, 595–602.
8. Allwood, M. A., Handwerger, K., Kivlighan, K. T., Granger, D. A., & Stroud, L. R. (2011). Direct and moderating links of salivary alpha-amylase and cortisol stress-reactivity to youth behavioral and emotional adjustment. *Biological Psychology*, 88(1), 57–64.
9. Kang, Y. (2010). Psychological stress-induced changes in salivary alpha-amylase and adrenergic activity. *Nursing & Health Sciences*, 12(4), 477–484.
10. Rohleder, N., Wolf, J. M., Maldonado, E. F., & Kirschbaum, C. (2006). The psychosocial stress-induced increase in salivary alpha-amylase is independent of saliva flow rate. *Psychophysiol.*, 43(6), 645–652.
11. Ceglarek, U., Werner, M., Kortz, L., Körner, A., Kiess, W., Thiery, J., & Kratzsch, J. (2010). Preclinical challenges in steroid analysis of human samples. *Journal of Steroid Biochemistry and Molecular Biology*, 121, 505–512.
12. Manickam P., Fernandez R.E., Umasankar Y., Gurusamy M., Arizaleta F., Urizar G., Bhansali S. (2018). Salivary cortisol analysis using metalloporphyrins and multi-walled carbon nanotubes nanocomposite functionalized electrodes. *Sensors and Actuator. B Chem.* 274 10.1016/j.snb.2018.07.133
13. Kim, I.-H., Kim, C., Seong, K., Hur, M.-H., Lim, H. M., & Lee, M. S. (2012). Essential oil inhalation on blood pressure and salivary cortisol levels in prehypertensive and hypertensive subjects. *Evidence-Based Complementary and Alternative Medicine*, 2012, 984203. <https://doi.org/10.1155/2012/984203>
14. Castro, M., Elias, P. C. L., Martinelli, C. R., Jr., Antonini, S. R. R., Santiago, L., & Moreira, A. C. (2000). Salivary cortisol as a tool for physiological studies and diagnostic strategies. *Brazilian Journal of Medical and Biological Research*, 33(10), 1171–1175.
15. Kokbas, U., Kayın, L., & Tuli, A. (2013). Biosensors and their medical applications. *Arch Med Rev J.*, 23, 499–513.
16. DündarYenilmez, E., Kökbaş, U., Kartlaşmış, K., Kayın, L., & Tuli, A. (2018). A new biosensor for noninvasive determination of fetal RHD status in maternal blood of RhD negative pregnant women. *PLOS ONE*, 13(6), e0197855.
17. Chavez, R. G., et al. (1986). An aromatic substituted glycoside. *American patent.*, 4(963), 479.
18. Moore, A., Raitken, R., Burke, C., Gaskell, S., Groom, G., Holder, G., Selby, C., & Wood, P. (1985). Cortisol assays: Guidelines for the provision of a clinical biochemistry service. *Annals of Clinical Biochemistry*, V22, 435–454.
19. Approved Guideline - Procedures for the Handling and Processing of Blood Specimens for Common Laboratory Tests, GP44-A4. (2010). Clinical and Laboratory Standards Institute