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Stability of salivary cortisol, alpha-amylase, and chromogranin A after long-term storage.

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Abstract

Background: The presence of stress biomarkers in saliva provides a convenient target to study body response in various conditions as well as in biobanking research. However, there are many conditions that may affect the assessment of these markers. Currently, there is no consensus over how long the saliva can be stored where the values of these markers remain stable. This study aims to investigate whether storage duration affects the measured concentrations of salivary cortisol (sCort), alpha-amylase (sAA), and chromogranin A (sCgA).

Methods: Saliva samples were measured for sCort levels using an enzyme immunoassay kit, for sAA activity using a portable biosensor, and for sCgA levels using an ELISA kit soon after collection. Remeasurement was performed after storage at -80 ºC for 3, 3.5, and 4 years (n= 15, 10, 15, respectively).

Results: The remeasured values of sCort and sAA were not different from the original measurement at all time points (p=0.221 and p=0.861, respectively). Surprisingly, the remeasured values of sCgA significantly increased compared with the original measurement ($p < 0.000$).

Conclusions: No significant changes were observed for sCort and sAA values after up to 4 years of storage while long-term storage of sCgA is not recommended as values would greatly differ from immediate measurement.

Keywords: saliva, cortisol, alpha-amylase, chromogranin A, storage, stability

Introduction

Saliva has become an increasingly valuable biological fluid in research and clinical practice due to its non-invasive collection compared with blood sampling [1]. It is also convenient and easy to handle since the subjects can collect the saliva by themselves. There are many markers that can be detected in saliva that reflect physiological, psychological, and pathological states. Among these, cortisol is one of the most studied and is considered the gold standard for stress marker [2] as it is a key indicator of the hypothalamic-pituitary-adrenal (HPA) axis activity [3]. Another commonly studied axis of stress response is the sympathetic-adrenal-medullary (SAM) system where alpha-amylase is the most studied surrogate marker [4]. Chromogranin A, a protein co-released with catecholamines during stress has also been gaining attention as a potential SAM biomarker [5].

A small number of studies have examined collection methods, handling conditions, storage conditions, as well as freezing/thawing impact on the levels of these biomarkers. For salivary cortisol (sCort), it was consistently found that ambient temperature did not affect the values of sCort for up to 5 days [6, 7]. The samples remained stable after three months of storage at 5^{o}C. Freezing the samples at -80 ^{o}C extended the stability up to one year with the enzyme immunoassay method [8] and up to six years with liquid chromatography tandem mass spectrometry method [9] and that repeated freezing/thawing had no effect [8].

For salivary alpha-amylase (sAA), there are even fewer studies examining the stability across different storage conditions with some inconsistent results [10-13]. For salivary chromogranin A (sCgA), there is no study examining such variables in humans. The stability of these biomarkers during storage is crucial,

particularly for long-term studies and biobanking initiatives. This gap in knowledge is particularly critical for researchers relying on archived saliva samples, where degradation over time could bias results or lead to underestimation of biomarker levels. Currently, there is no clear consensus on how long each of these biomarkers remains reliably quantifiable.

In this study, we aim to fill the gap by comparing the effect of long-term storage on sCort, sAA, and sCgA. We employed the samples we have assayed immediately after collection and remeasured after storage at 3, 3.5, and 4 years under a real-world condition of storage at -80 ºC. By assessing whether analyte concentrations remain within accepted limits over time, this work will provide guidance on best practices for saliva biobanking and the validity of data derived from archived samples.

Methods

Saliva samples

The saliva samples were originally from our two previous experiments [14, 15]. The samples were chosen based on the duration of storage and that there was enough amount of saliva left for remeasurement of the biomarkers. The original identification of the participants who provided the saliva was unknown as all samples were coded in storage. The saliva was collected in Jan 2019 for a 4-year-old samples, July 2019 for a 3.5-year-old samples, and Jan 2020 for a 3-year-old samples. The saliva samples were kept in a 2-mL microcentrifuge plastic tube and stored at 4 ºC for no more than a day before storage at -80 ºC after the first collection. The samples were later thawed and measured in the original experiments. After that, they were kept at -80 ºC throughout without thawing or refreezing for all of the samples. The remeasurement was performed in Jan 2023 for sCort and sAA and in Feb 2023 for sCgA (3-4 years after first collection of saliva).

Measurement of salivary biomarkers

The levels of biomarkers were measured as previously described in the original experiments [14, 15]. The frozen saliva was thawed and centrifuged at 1500g for 15 min. The supernatant was retrieved for measuring sCort, sAA, and sCgA levels. The levels of sCort were measured using a competitive enzyme immunoassay (EIA) kit (Salimetrics, State College, PA, USA) according to the manufacturer's protocol. The sensitivity of the kit can measure between 0.007 to 3 µg/dL sCort. The intra- and inter-assay coefficients of variation were 7% and 11%, respectively. The levels of sAA were measured with a portable amylase biosensor (Nipro Co, Osaka, Japan) as previously described [16]. Briefly, an

aliquot of 25 µL saliva was put onto the detector's strip and inserted to be read in a portable biosensor. It was recommended that the biosensor be used to measure sAA levels up to 200 U/mL. The coefficient of variation was 8.4% [17]. The levels of sCgA were measured with an ELISA kit according to the manufacturer's protocol (MyBioSource, San Diego, CA, USA). The detection range of the kit was 30-9,000 ng/L with the lowest sensitivity at 15.21 ng/L. Both the intra- and inter-assay precision were less than 10%. For sCgA, the 4-year-old samples were measured with a kit from a different company so only the 3- and 3.5-year-old samples were assessed for sCgA levels.

Statistical analysis

Data were presented as mean ± SEM unless stated otherwise. Kolmogorov-Smirnov test was used to determine normality of the samples. All analyses were performed with nonparametric tests and included all samples (no outliers were omitted). Wilcoxon signed-rank test was used to compare the means of the original biomarkers' levels and the remeasured levels. Agreements between different storage durations' effect on the biomarkers' values were analyzed using the Bland-Altman plots with log-transformation. Statistical analysis was performed using SPSS statistics program version 21 (IBM, New York).

Results

The original samples were collected in Jan 2019, Jul 2019, and Jan 2023 and were remeasured in Jan-Feb 2023, making the storage durations to be 3, 3.5, and 4 years for sCort and sAA. The sCgA samples were 3 and 3.5 years only. The data were not normally distributed according to the Kolmogorov-Smirnov test. The mean ± SEM of the original values of three salivary biomarkers and the remeasured ones from all three time points are shown in Table 1 (and in Supplementary Figure 1 for values at each time points). Storing the saliva at -80 ^{o}C for up to 4 years did not affect the values of sCort (p=0.221) and sAA (p=0.861) according to the Wilcoxon signed-rank test. However, the values of sCgA were actually higher after 3 and 3.5 years of storage (p<0.000). Interestingly, the SEM for sCort and sAA were also similar between original and remeasured values, while that of remeasured sCgA were much higher than the original values.

The comparison of the percentage change of the three biomarkers is shown in Figure 1A. For sCort, the average changes were -2.42 ± 12.53, -0.16 ± 11.77, and 18.08 ± 15.88 % at 3, 3.5, and 4 years respectively, compared with the original values. For sAA, the values were more varied showing average changes at 75.10 ± 35.17, 13.66 ± 12.67, and -13.23 ± 10.01 % at 3, 3.5, and 4 years respectively, compared with the original values. These changes were most pronounced with sCgA where the average changes were 176.5 ± 42.46 and 162.4 ± 87.37 % at 3 and 3.5 years, compared with the original values. The value changes from original measurement for sCort, sAA, and sCgA at 3, 3.5, and 4 years were also shown for comparison (Figure 1B).

The Bland-Altman plots of agreement between original and remeasured values were performed and showed good agreement for sCort and sAA (Figure 2). While that of sCgA was not as good compared with the other two biomarkers.

Of note is that we did have a 4-year-old samples of sCgA. However, the original and the new measurement was performed using kits from different companies and were not actually comparable. We tried measuring out of curiosity and found that the values of sCgA from 4-year-old samples were within the same ranges as the 3- and 3.5-year-old samples (Supplementary Table 1). However, when compared with the original values measured by a different kit company, we had to convert the original values (pmol/mL and pmol/mg protein) to ng/mL. This led to the original values being very high. The remeasured values were only 15.32% of the original values.

Table 1. Mean levels of salivary biomarkers (± SEM) after storage for 3-4 years.

Markers	**Original values**	**Remeasured values**	***p*-value**
sCort (µg/dL)	0.277 ± 0.038	0.268 ± 0.039	0.221
sAA (U/mL)	97.80 ± 12.21	97.60 ± 12.65	0.861
sCgA (ng/L)	1179.88 ± 135.24	3246.43 ± 670.89	0.000

n=40 for sCort and sAA from 3, 3.5, and 4 y samples, n=24 for sCgA from 3 and 3.5 y samples.
Wilcoxon signed-rank test was used to compare original and remeasured values.
sCort, salivary cortisol; sAA, salivary alpha-amylase; sCgA, salivary chromogranin A

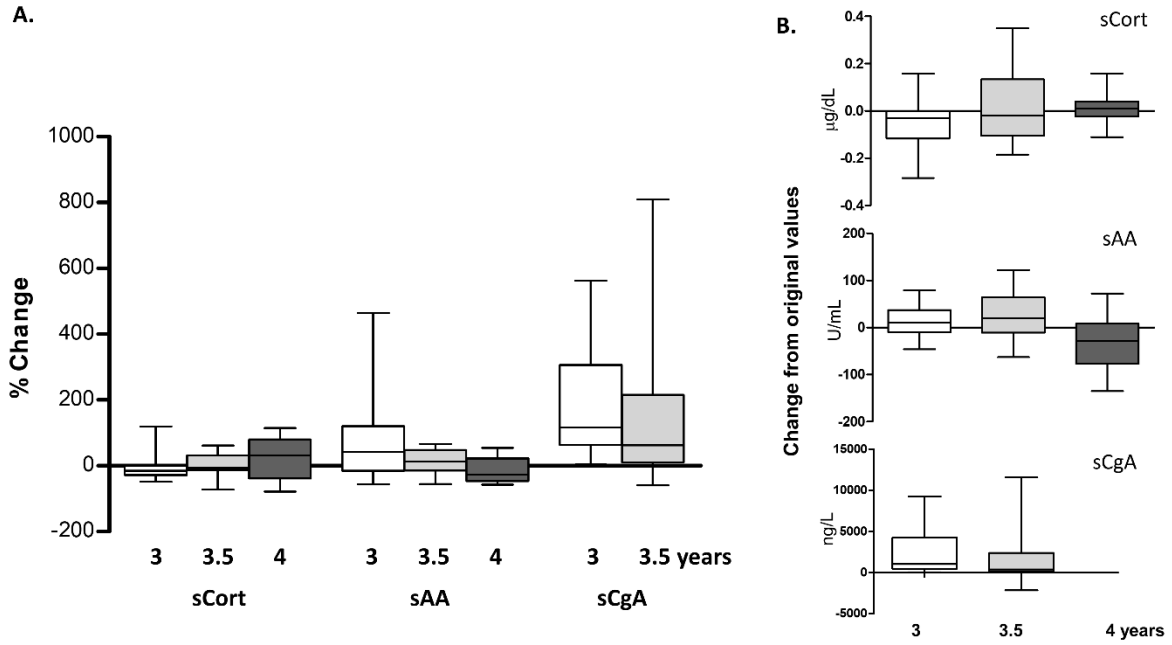

Figure 1. A) The percentage change of biomarkers' values compared with original measurement after storage for 3, 3.5, and 4 years. B) The value change from the original measurement. n=15, 10, 15 for 3, 3.5, 4 y samples, respectively for sCort and sAA; n=14, 10 for 3, 3.5 y samples for sCgA. sCort, salivary cortisol; sAA, salivary alpha-amylase; sCgA, salivary chromogranin A

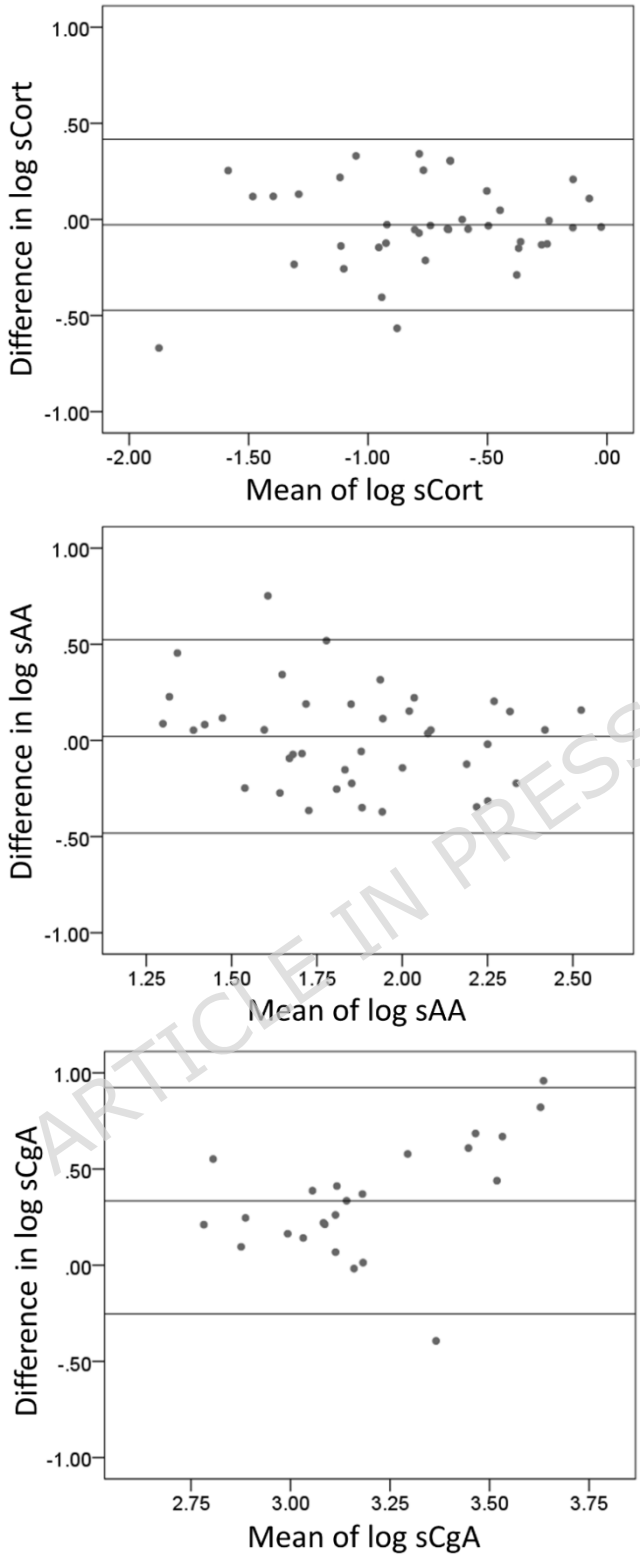

Figure 2. The Bland-Altman plots of agreement between original and remeasured values of sCort, sAA, and sCgA. The horizontal lines indicate mean ± 1.96 SD. n=40 for sCort and sAA, n=24 for sCgA from all time points. sCort, salivary cortisol; sAA, salivary alpha-amylase; sCgA, salivary chromogranin A.

Discussion

This study compared the stability of three common salivary biomarkers used in stress research: sCort, sAA, and sCgA. This is a real-world investigation after storage in the laboratory under natural condition at -80 ºC. The results demonstrate that for sCort and sAA, the values remained stable showing no significant changes across all storage durations of up to four years. In contrast, the values of sCgA exhibited clear evidence of variation over time, suggesting that sCgA is not suitable for long-term storage or for biobanking research.

The observed stability of sCort aligns with earlier studies reporting no change in sCort levels stored for up to a year with EIA method as in the current study and up to six years with LC/MS method at deep freeze temperature [8, 9]. Our results strengthened the finding that with the EIA method, measurement of sCort is still reliable up to four years. This reinforces the notion that the steroid hormone, due to its chemical structure and relative resistance to enzymatic breakdown, is highly stable in saliva.

For sAA, the current study provides a new piece of evidence that under optimal temperature, sAA remained stable for four years. This is the first study to examine such long-term storage effect on sAA values. Previous studies have shown inconsistent results. One study explored different temperature conditions at 4, 20, 30, and 40 ºC for up to one month and found that sAA activity reduced in relation to higher temperature or repeated freeze-thaw cycles [10]. While other studies found sAA to be stable at room temperature for up to ten days without impact from repeated freezing/thawing or at -80 ºC for up to six months [11-13]. The Bland-Altman plot for sAA shows a comparable window of difference compared with our previous study that examined the measurement of sAA using different methods [16]. Further supporting for the reliability of measuring sCort

and sAA were the similarity not only in the mean values but also in the deviations of these values (as shown with similar SD or SEM). Our findings extend these observations by showing that the stability of sAA persists even at four years of storage, supporting its utility in long-term biobanking projects.

In contrast, sCgA appeared unstable over time. Surprisingly, sCgA levels actually increased after long-term storage. Such increases were not different between 3- and 3.5-year-old samples (Figure 1). The reason behind this increase is unknown. Previous studies have also shown increased levels of various analytes after storage such as hydrogen peroxide and adenosine deaminase [13]. The techniques used to measure these markers might need to be further elucidated and standardized. Dilution of samples might also be one factor that could make the results more variable. For sCgA, the values of the biomarker in saliva or in other biological fluids can vary over a very large range of values beyond the detection limit of the particular assay. It may take a while to find the optimal dilutional factor for each sample set. While for sCort and sAA, dilution of samples was not necessary making it easy and convenient to assay.

Of note is the comparison of 4-year-old sCgA values using a different kit company. It was found that this method is highly not recommended as the unit of measurement was different (ng/L vs pmol/mL). The values were totally different in that the remeasured values were only about 15% that of original values (Supplementary table 1) which were in contrast to the 3-year-old values where remeasured values were higher than the original ones. It is possible that the kits used antibodies that bind to different sequences of CgA protein and whether the antibodies can also bind to CgA fragments/metabolites was not known. This suggests the standardization of the protocol is still needed and that using different kits are not recommended.

These findings have several implications. First, they support the validity of using biobanked saliva samples for retrospective analyses of sCort and sAA, even when samples have been stored for up to four years. Second, they highlight the need for methodological adjustments when studying sCgA, such as limiting the storage period or analyzing samples as soon as possible after collection. Finally, they underscore the importance of reporting storage conditions and durations in biomarker studies, as these factors may influence the reliability of certain analytes.

This study has some limitations that should be acknowledged. We analyzed samples stored under a single set of conditions (−80 °C, no freeze-thaw cycles), and results may differ under other storage temperatures or handling protocols. The 4-year-old sCgA samples were measured with different kits and cannot be directly compared. Also, the biological activity of sCgA was not determined. Future work should explore strategies to preserve unstable protein biomarkers such as sCgA and that standardization of the protocol is greatly needed as this could help in comparison of results from different laboratories.

In conclusion, sCort and sAA demonstrate excellent long-term stability in samples stored at -80 °C for up to four years, supporting their use in biobanking and retrospective studies. By contrast, sCgA is unstable over time, and caution is warranted when analyzing long-stored samples. These findings provide important guidance for researchers relying on archived saliva samples in stress and psychobiological research.

Abbreviations

EIA: enzyme immunoassay

ELISA: enzyme-linked immunosorbent assay

HPA: hypothalamic–pituitary–adrenal

sAA: salivary alpha-amylase

sCgA: salivary chromograinin A

sCort: salivary cortisol

SAM: sympathetico-adrenal-medullary

Declarations

Ethics approval and consent to participate

The original study protocols were approved by Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Institutional Review Board COA. No. MU-DT/PY-IRB 2017/065.1812 and MU-DT/PY-IRB 2019/045.0907. The trials were registered in thaiclinicaltrials.org, registration number TCTR20180503002 and TCTR20190801001. The study was conducted according to the principles of the Declaration of Helsinki. All participants gave written informed consent.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

P.P. conceptualized the study, analyzed and interpreted the data, and finalized the manuscript. N.J. performed the experiment, analyzed the data, and drafted and finalized the manuscript. All authors read and approved the final manuscript.

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