ORIGINAL ARTICLE

Measurement of steroid hormones in saliva: Effects of sample storage condition

REBECCA J. TOONE1, OLIVER J. PEACOCK1, ALAN A. SMITH1, DYLAN THOMPSON1, SCOTT DRAWER2, CHRISTIAN COOK1,2 & KEITH A. STOKES1

1 University of Bath: Sport, Health and Exercise Science, Department for Health, University of Bath, Bath, and
2 UK Sports Council, London, UK

Abstract
Measurement of steroid hormones in saliva is increasingly common in elite sport settings. However, this environment may enforce handling and storage practices that introduce error in measurement of hormone concentrations. We assessed the influence of storage temperature and duration on reproducibility of salivary steroid levels. Nine healthy adults provided morning and afternoon saliva samples on two separate occasions. Each sample was divided into identical saliva aliquots which were stored long-term (i.e. 28 and 84 days) at 80°C or 20°C (testing day 1), and short-term (i.e. 1, 3, 7 and 14 days) at 4°C or 20°C (testing day 2). Samples were analyzed for cortisol, testosterone and estradiol using ELISA. In non-freezer conditions, there was a decrease from baseline to 7 days in testosterone (26 ± 15%) and estradiol (58 ± 17%) but not cortisol concentrations (p < 0.001). This decrease was larger in samples stored at room temperature than in the refrigerator (p ≤ 0.01). There were small but significant changes in measured concentrations of all hormones after 28 and/or 84 days of storage in freezer conditions (p ≤ 0.01), but these were generally within 12% of baseline concentrations, and may be partly explained by inter-assay variability. Whole saliva samples to be analyzed for cortisol, testosterone and estradiol should be frozen at 20°C or below within 24 h of collection, and analyzed within 28 days. Storage of samples for measurement of testosterone and estradiol at temperatures above 20°C can introduce large error variance to measured concentrations.

Key Words: Cortisol, estradiol, saliva hormones, sample storage, steroids, testosterone

Introduction
Assessment of hormone responses to athletic activity can provide valuable information regarding training stress, adaptation and exercise performance [1]. Measurement of steroid hormones in saliva is often used as an alternative to blood in studies of elite performers [2–8]. Saliva collection procedures are non-invasive, easy to administer, stress-free and enable repeated sampling over the course of minutes or days [9]. Furthermore, validation studies have demonstrated good agreement between salivary and total serum hormone concentrations [7]; especially the free or biologically active fraction of blood hormones [7,10,11].

Factors such as storage duration and temperature can influence saliva concentrations of steroid hormones because of degradation by enzymes within the saliva matrix [12,13], while microorganisms in the oral cavity are potent decomposers of salivary components [14,15]. Cortisol has been reported to be stable at 5°C for up to 3 months or at −20°C and −80°C for up to one year, but to decrease by approximately 9% per month in samples stored at room temperature [16]. In contrast, others have reported a 25% decrease in salivary cortisol after just 3 weeks of storage at 4°C or at room temperature, and an alarming 30–70% decrease over this period in samples that were not centrifuged prior to storage [17]. Testosterone has been reported to be stable at −80°C for up to three years but to decrease by 18% after 6 months at −20°C and increase by 21% after 1 week and 331% after 4 weeks of storage at 4°C [9]. Despite these mixed findings, the general consensus communicated in recent review articles is that salivary...
steroids are stable for up to 1 month at 4°C (i.e. in a regular household refrigerator) and for up to 3 months at −20°C [18–20].

Saliva collection and analysis for the measurement of hormone concentrations is becoming increasingly routine in elite sport. However, this setting places restrictions on how samples can be handled and stored after collection, especially in relation to access to freezers and centrifuges. As such, samples may be exposed to storage for long periods at room or fridge temperature (e.g. across training camps or during transportation) as whole native saliva before delivery to the laboratory. Therefore, the purpose of this study was to assess the effect of storage condition and duration on the concentration of selected steroid hormones typically analyzed in elite sport. Specifically, we investigated the influence of storage temperature at 4°C compared to 20°C on salivary cortisol, testosterone and estradiol concentrations after 1, 3, 7 and 14 days; and the longer term (28°C) stability of these hormones in whole saliva when stored in freezer conditions (−20°C and −80°C).

**Materials and methods**

**Participants**

Saliva samples were obtained from healthy male (n = 6) and female (n = 3) adult volunteers aged 21–39 years. Ethical approval was granted by the local National Health Service Research Ethics Committee, in line with the Helsinki Declaration of 1975.

**Preliminary investigation**

An initial pilot study was used to inform the methods used in the present investigation. Specifically, we aimed to determine whether taking multiple aliquots sequentially from the same sample would impact upon measured concentrations in each aliquot. Participants (n = 2) were asked to produce three consecutive 6 mL saliva samples into 10 mL containers. Participants were able to provide the 18 mL of saliva within 15–30 min. The entire 6 mL of one sample, and half (3 mL) of the other two samples were vortexed and split into 1-mL aliquots. The aliquots were frozen at −80°C and stored overnight before analysis of testosterone and cortisol by enzyme-linked immunosorbent assay (ELISA; Salimetrics, PA, USA). Measured mean concentrations (mean ± SD) for all six aliquots for the two participants were 0.26 ± 0.01 nmol·l⁻¹ and 0.17 ± 0.01 nmol·l⁻¹ for testosterone, and 2.7 ± 0.3 nmol·l⁻¹ and 3.8 ± 0.3 nmol·l⁻¹ for cortisol. Coefficient of variation was calculated for each hormone for each participant and all fell in the range 4–11%, indicating that measured hormone concentrations were not influenced by sequential aliquoting.

**Experimental procedures**

To study the short-term effects of sample storage in a regular household refrigerator compared to room temperature (i.e. conditions indicative of those typically available at field-based testing sites in elite athletes) and the longer duration stability of hormones in samples stored in freezer conditions (i.e. −20°C and −80°C), participants provided saliva samples on two separate testing days (separated by 1 week). On each testing day, participants provided two 10 mL saliva samples via unstimulated passive drool into 15 mL collection tubes with no additive. Samples were collected on separate days to allow the volume required to be collected within a reasonable time. In order to achieve a wide range of hormone concentrations, we took advantage of the circadian variation in hormones by collecting samples from all participants both upon waking and in the late afternoon on each testing day. As such, we collected a total of 18 different samples over a range of concentrations (testosterone: 0.06–0.50 nmol·l⁻¹; estradiol: 0.010–0.038 nmol·l⁻¹; cortisol: 1.4–21.0 nmol·l⁻¹) for storage in each condition. To minimize any contamination of samples, participants were asked to refrain from brushing their teeth and eating or drinking in the 60 min prior to sample collection. Participants were also required to rinse their mouths with water 10 min prior to sampling.

Following collection, samples were vortexed prior to being dispensed into eight separate 1-mL aliquots and stored in the appropriate condition. One aliquot of each of the 18 samples were stored for the required number of days in each condition. On testing day 1, one aliquot was frozen at −80°C and analyzed after 24 h of storage (baseline). Further aliquots were frozen at −80°C and −20°C and analyzed after 28 days and 84 days. On testing day 2, one aliquot was frozen at −80°C and analyzed after 24 h of storage (baseline). Further aliquots were stored in a refrigerator at 4°C or at a room temperature of 20°C, and were analyzed after 1, 3, 7 and 14 days. For consistency in the sequence of aliquoting and assay procedures, all −80°C samples were handled prior to −20°C samples, and all 4°C samples were handled prior to those stored at 20°C. On all occasions, morning samples were processed prior to afternoon samples. The preliminary investigation demonstrated no significant difference in concentration across sequential pipetting of samples, suggesting that it is unlikely that the aliquoting order used introduced systematic error.

Once samples had been stored for the designated amount of time in the assigned condition, all samples (stored at a higher temperature than −80°C) were...
transferred to −80°C overnight in order that samples stored at room and fridge temperature completed a freeze-thaw cycle to precipitate the mucins in the sample [21] and so that all samples could be defrosted from the same condition. Following overnight freezing at −80°C, samples were defrosted at room temperature, centrifuged for 10 min at 5000 rpm in a micro-centrifuge to remove particulate matter before clear sample was removed and pipetted into separate Eppendorf tubes. Samples were analyzed in duplicate for concentrations of testosterone, cortisol, and estradiol by ELISA (Salimetrics, PA, USA). The intra-assay co-efficient of variation was less than 9% for all assays. Inter-assay reliability calculated using the same high and low control samples run in quadruplicate on all plates were 5.4 and 8.4% for cortisol (high control: 26.8 nmol·l⁻¹, low control: 2.7 nmol·l⁻¹), 11.8 and 18.3% for testosterone (high control: 0.58 nmol·l⁻¹, low control: 0.06 nmol·l⁻¹), and 17.6 and 16.5% for estradiol (high control: 0.094 nmol·l⁻¹, low control: 0.020 nmol·l⁻¹). For all hormones, the concentrations of the low and high controls were of a similar range to measured values. None of the measured concentrations were below the limits of detection (cortisol: <0.008 nmol·l⁻¹; testosterone: 0.003 nmol·l⁻¹; estradiol: 0.0004 nmol·l⁻¹).

Assay performance characteristics for each kit were accepted as those reported by the manufacturer in the kit insert.

Statistical analysis

A two-way general linear model for repeated measures (treatment × time) was used to identify overall differences between experimental conditions. The Greenhouse-Geisser correction was used for epsilon <0.75 and the Huynh-Feldt correction was adopted for less severe asphericity. Where significant F values were found, the Holm-Bonferroni stepwise correction was applied to determine the location of variance. All data are expressed as mean ± standard deviation. Statistical significance was set at the 0.05 level. The likelihood that any observed changes in a given sample were influenced inter-assay variability was calculated using the formulae of Reed et al. [22], from the quadruplicate samples of control material on each plate. The formula allows calculation of the probability that the difference between two samples is due to inter-assay variability. A CV was calculated for both low and high control material, providing a probability range over which the change in concentration may be due to inter-assay variation. The CVs calculated for high and low controls, respectively, were 11.8 and 18.3% for testosterone, 17.6 and 16.5% for estradiol, and 5.4 and 8.4% for cortisol. Assay CV was entered into the formula of Reed et al. [22] along with measured changes in concentration for samples at each time-point in each storage condition.

Results

Cortisol

Baseline cortisol concentration for the freezer conditions was (mean ± SD) 6.9 ± 5.8 nmol·l⁻¹ (range: 1.4–19.0 nmol·l⁻¹). Concentrations were not different between −80°C and −20°C conditions after 28 or 84 days of storage. Following 28 days (6.3 ± 5.2 nmol·l⁻¹, p < 0.01) and 84 days (6.3 ± 5.0 nmol·l⁻¹, p < 0.01) of storage, concentrations were significantly lower than baseline. This represented a mean decrease of 9 ± 11% (p < 0.01) and 10 ± 13% (p < 0.01), respectively. Applying the Reed formula indicates that there is a 34% and 36% probability that decrease in cortisol concentrations observed after 28 and 84 days of storage, respectively, was due to inter-assay variation.

Under non-freezer conditions, baseline cortisol concentration was 7.7 ± 5.2 nmol·l⁻¹ (mean ± SD) (range: 1.4–17.4 nmol·l⁻¹). Values did not change significantly from this baseline value, and there were no differences between the non-freezer conditions in either the magnitude or the time-course of these changes (Figure 1).

Testosterone

Baseline testosterone concentration for the freezer conditions was 0.34 ± 0.11 nmol·l⁻¹ (range: 0.12–0.52 nmol·l⁻¹). Concentrations were not different between −80°C and −20°C conditions after 28 or 84 days of storage. There was a main effect for time, and concentrations after 84 days of storage (0.42 ± 0.13 nmol·l⁻¹, p < 0.001) were significantly greater than both baseline and day 28 concentrations (Figure 2). This represented a mean increase of 24 ± 16% (P < 0.001) above baseline.

Figure 1. Mean saliva cortisol concentrations (with 95% confidence intervals for the change in concentration over time) for storage at −80°C, −20°C, 4°C and at room temperature (20°C). #, Significantly different from baseline across both treatments (n = 18).
In the non-freezer storage conditions, baseline concentration was $0.33 \pm 0.14 \text{ nmol} \cdot \text{l}^{-1}$ (range: $0.07$–$0.58 \text{ nmol} \cdot \text{l}^{-1}$). There was an interaction effect, with significant decreases in testosterone concentrations in $4\degree \text{C}$ conditions (day 3, $0.31 \pm 0.15 \text{ nmol} \cdot \text{l}^{-1}$; day 7, $0.27 \pm 0.13 \text{ nmol} \cdot \text{l}^{-1}$, both $p < 0.01$) and room temperature conditions (day 1, $0.28 \pm 0.13 \text{ nmol} \cdot \text{l}^{-1}$; day 3, $77 \pm 36 \text{ pg} \cdot \text{ml}^{-1}$; day 7, $69 \pm 36 \text{ pg} \cdot \text{ml}^{-1}$, all $p < 0.001$). Using the Reed formula, there is a $27\%$ probability that the mean $26 \pm 15\%$ ($p < 0.001$) decrease in testosterone concentrations on day 7 was due to inter-assay variation. In both $4\degree \text{C}$ ($0.37 \pm 0.16 \text{ nmol} \cdot \text{l}^{-1}$) and room temperature ($0.33 \pm 0.14 \text{ nmol} \cdot \text{l}^{-1}$) conditions, concentrations had returned to near baseline concentrations after 14 days ($0.35 \pm 0.15 \text{ nmol} \cdot \text{l}^{-1}$). Testosterone concentrations across days 1–14 were significantly lower when samples were stored at room temperature compared with $4\degree \text{C}$ ($p < 0.001$; Figure 2).

**Estradiol**

Baseline estradiol concentration under freezer conditions was $0.018 \pm 0.006 \text{ nmol} \cdot \text{l}^{-1}$ (range: $0.009$–$0.029 \text{ nmol} \cdot \text{l}^{-1}$). There was a main effect for time, and following 28 days of storage concentrations were lower than baseline ($0.016 \pm 0.005 \text{ nmol} \cdot \text{l}^{-1}$, $p < 0.01$), representing a decrease of $9 \pm 12\%$ ($p < 0.01$), although application of the Reed formula suggests that this decrease was likely (72% probability) to be due to inter-assay variation. There were no differences between $-80\degree \text{C}$ and $-20\degree \text{C}$ treatments at any time-point.

Baseline estradiol concentration in non-freezer conditions was $0.019 \pm 0.007 \text{ nmol} \cdot \text{l}^{-1}$ (range: $0.010$–$0.035 \text{ nmol} \cdot \text{l}^{-1}$). There was an interaction effect, with significant decreases in estradiol concentrations in $4\degree \text{C}$ conditions (day 3, $0.014 \pm 0.005 \text{ nmol} \cdot \text{l}^{-1}$; day 7, $0.009 \pm 0.004 \text{ nmol} \cdot \text{l}^{-1}$, day 7, $0.014 \pm 0.005 \text{ nmol} \cdot \text{l}^{-1}$, all $p < 0.001$) and room temperature conditions (day 1, $0.014 \pm 0.007 \text{ nmol} \cdot \text{l}^{-1}$; day 3, $0.012 \pm 0.006 \text{ nmol} \cdot \text{l}^{-1}$; day 7, $0.007 \pm 0.004 \text{ nmol} \cdot \text{l}^{-1}$, day 7, $0.013 \pm 0.006 \text{ nmol} \cdot \text{l}^{-1}$, all $p < 0.001$). At 7 days, the mean decrease from baseline in non-freezer conditions was $58 \pm 17\%$ and based on the Reed formula there is a $6\%$ probability that this decrease was due to inter-assay variance. Estradiol concentrations on days 1 and 3 were significantly lower when samples were stored at room temperature compared with $4\degree \text{C}$ ($p < 0.001$; Figure 3).

**Discussion**

The main focus of the present study was to assess the effects of storage temperature and duration on measured concentrations of steroid hormones in whole, native saliva. Cortisol concentrations were not different between aliquots frozen immediately and those stored at room temperature or in a refrigerator for up to 7 days. In contrast, there was a decrease in testosterone and estradiol concentrations across 1–7 days of storage in non-freezer conditions; but samples stored in a refrigerator were more stable than those stored at room temperature. Storage of samples in freezer conditions for up to 84 days resulted in concentrations typically within 12% of baseline levels for all hormones, and further analysis using the formula of Reed et al. [22] revealed a large proportion of these changes can be attributed to inter-assay variance.

In the present study, there were no effects on the concentrations of salivary cortisol after storage of samples for up to 7 days in a refrigerator or at room...
temperature. This finding is consistent with reports that cortisol is stable for up to 1 week at 4°C and at 18°C in whole native saliva [17]. In contrast, the data reported here shows a decrease in testosterone and estradiol concentrations across this period, reaching a mean low of 26 ± 15% and 58 ± 17% below baseline at 7 days, respectively. This degradation in sample concentrations was shown to be significantly more pronounced in samples stored at 20°C than at 4°C, for testosterone (at all time-points) and estradiol (at days 1 and 3). These observations are in agreement with previous research showing no differences in cortisol levels after 4 days storage in non-freezer conditions, but a marked reduction in testosterone and estradiol concentrations after 4 days in the refrigerator (18% and 34%) or at room temperature (32% and 64%), respectively [23]. One plausible explanation is the influence of bacteria on sample integrity, given previous evidence of a decrease in testosterone but not cortisol in samples loaded with bacteria and when stored at room temperature prior to analysis [24]. Thus, our data support the notion that bacterial multiplication may contribute to the decrease in gonadal steroid hormone concentrations during storage at higher temperatures.

Following the marked decrease in measured testosterone and estradiol levels after 1 week of storage in non-freezer conditions, the subsequent increase or ‘rebound’ in the concentrations of these hormones reported after 2 weeks was unexpected and, to our knowledge, this pattern has not been reported previously. Other authors have observed an increase in testosterone concentrations in samples stored at 4°C, but this was a linear increase from initial concentrations, and was determined using a single pooled sample from all participants [9]. The increase in concentrations from day 7 to day 14 in the present study was systematic, with nearly all individual samples demonstrating an increase in concentrations. This effect cannot be explained by assay variation, as control values did not demonstrate the same pattern. It is beyond the scope of this study to determine the reason for this effect, but possible explanations include changes in sample pH, enzymatic activity, bacterial multiplication, and hydrolytic cleavage of steroid conjugates in free forms of the steroid. Where the pH of the sample drops below 4, the antibody-antigen reaction necessary for accurate measurement of salivary biomarkers by immunoassay is compromised, resulting in artificially high estimates of hormone concentrations [21]. In urine samples, microbial contamination can induce modification of steroid structure by oxidoreductive reactions [25]. As such, while an increase in steroid concentrations due to bacterial growth is less frequently observed than a decrease, an increase has been reported to occur in urine due to bacterial hydrolysis of androstenediol, followed by 3-beta hydroxysteroid dehydrogenase and steroid isomerase activity [26]. This may also apply to estradiol given that conversion from androstenedione also occurs via the action of 3-beta hydroxysteroid dehydrogenase. Hydrolytic cleavage of steroid conjugates resulting in an increase in free steroid concentration could also have produced the results demonstrated here. Certain bacteria and enzymes can cause deconjugation or hydrolysis of the steroid from its glucuronide and sulfate forms [27], reactions that, similar to those above, is influenced by the amount of enzyme/bacteria, temperature, duration of incubation, and the pH [27]. Whether the specific enzymatic reactions mentioned above continue within frozen samples, and are responsible for the ‘rebound’ effect seen in testosterone and estradiol concentrations in the present investigation, warrants further investigation. However, these effects appear to be specific to testosterone and estradiol, and the longer duration seen before this effect as the temperature decreased may be indicative of temperature rate limited reactions.

It is noteworthy that the storage of steroid hormones in saliva was more stable in samples stored in freezer compared to non-freezer conditions, and that there were no significant differences between a regular house-hold freezer (−20°C) compared to storage at ultra-low temperatures (−80°C). Variation in cortisol concentrations was less than 10% for storage of samples for up to 3 months, but was shown to be significantly different from baseline after both 28 and 84 days. This is in contrast to the findings of others who have shown that salivary cortisol is stable for up to 1 year when archived at −20°C or −80°C [16] in centrifuged saliva. However, cell and cell fragments within the oral mucosa contained in whole saliva provide an optimal surface for bacterial growth [28], so centrifugation prior to storage likely preserves the concentration of hormones within the sample [9,17]. The present investigation aimed to preserve ecological validity by storing whole saliva as would be done in an exercise training environment; thereby providing greater real-world relevance. In a clinical setting, salivettes using a cotton swab are very often used in the collection of saliva. Future research could consider whether the results obtained in the present investigation remain while using this alternative collection method.

To the best of the authors’ knowledge, this is the first study to report on the stability of salivary estradiol concentrations in freezer conditions, and our data show small albeit significant decreases in estradiol concentrations (i.e. less than 10% from baseline) after 1 month, followed by a return to near baseline values by 3 months. Although testosterone concentrations were shown to be stable after 1 month in freezer conditions, a similar but more marked subsequent increase in concentrations to that observed for estradiol was shown at 3 months. This increase in gonadal steroid concentrations for samples stored in
freezer conditions is in contrast to others who have shown an 18% decrease or no change in measured testosterone levels when stored for 6 months at −20°C or −80°C, respectively [9]; but may be a function of differences between studies in the duration of sample storage prior to analysis.

It is important to highlight that potential sources of pre-analytical measurement error (such as drift in the quality and concentration of reagents and controls as well as subtle differences in assay technique) may account for some of the variance in hormone levels across time in the present study. As an expression of plate-to-plate consistency, the inter-assay coefficient of variation calculated from the mean values for the high and low controls on each plate was less than 20% for all analyses; which is considered to be acceptable for immunoassays [22,29]. Application of the formula of Reed et al. [22], as a method to further explore the probability that any observed changes in a given single sample were influenced by inter-assay variation suggests that changes in hormone concentrations across the period of storage needed to be fairly large to be attributable to storage condition per se. For example, there was approximately an 80% probability that the relatively small measured changes in gonadal steroids after 1 month storage in freezer conditions was due to inter-assay variation. In contrast, the likelihood that the relatively large decrease in testosterone or estradiol concentrations by 7 days of storage at room or fridge temperature was due to assay variance was 26% and 6%, respectively; thereby more likely representing a true effect of storage temperature as a source of measurement error.

The present results on the reliability of salivary steroid hormones under different storage conditions highlights problems that may occur when saliva is not frozen in the hours immediately after collection. As long as these changes remain within the precision of the assay, however, even significant differences are not practically meaningful. Nonetheless, greater changes in hormone concentrations associated with storage condition could be misinterpreted and may, for example, confuse the interpretation of the stress response to training in elite sport. The present findings suggest that, in order to preserve the 'original' concentrations of hormones in saliva, gonadal steroids should be kept refrigerated upon collection and stored at −20°C or below within 24 h. Samples to be analyzed for cortisol may be stored or transported for up to 7 days at room temperature or in a refrigerator before freezing. Samples to be analyzed for cortisol, testosterone or estradiol should probably be analyzed within one month of collection.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


