Chapter IX

Testosterone, androstenedione, cortisol and cortisone levels in human unstimulated, stimulated and parotid saliva

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Abstract

Background Recently measurements of steroids like testosterone, androstenedione, cortisol and cortisone in saliva are more and more applied in diagnostics and scientific studies. This is mainly due to the simple and non-invasive collection of saliva. We aimed to evaluate the optimal way to collect saliva for steroid hormone measurement.

Methods We investigated in twenty volunteers whether there is a difference between steroid hormone concentrations in unstimulated and stimulated saliva collected while chewing, using cotton and synthetic Salivettes®, citric acid or chewing gum. Furthermore, total unstimulated saliva was compared to parotid gland saliva. Testosterone, androstenedione, cortisol and cortisone were measured using Liquid-Chromatography Tandem Mass Spectrometry (LC-MS/MS).
Results Salivary testosterone, androstenedione and cortisol concentrations were unaffected by stimulation, cortisone levels were on average 16% lower. Concentrations of all hormones were lower in parotid gland saliva compared to unstimulated whole saliva (on average 51%, 26%, 66% and 49% lower, for testosterone, androstenedione, cortisol and cortisone, respectively). Concentrations of testosterone as well as androstenedione were lower when using synthetic Salivettes® (58% and 41%, respectively) and were higher when using cotton Salivettes® (217% and 46%, respectively). Cortisol levels in saliva were unaffected by using Salivettes®. However, cortisol and testosterone levels were lower in with chewing gum stimulated saliva (16% and 55%, respectively). Cortisone concentrations were lower in all types of stimulations (on average 25%-35%).

Conclusion The way saliva is collected should be taken into account when analysing and interpreting salivary hormone concentrations. We advocate unstimulated saliva collection in simple polypropylene tubes for all steroid measurements.

Introduction

In recent years, the measurement of steroid hormones like testosterone, androstenedione, cortisol and cortisone in saliva is more and more applied in both patient care and scientific studies. The primary advantage of saliva is that it offers non-invasive sampling. Saliva measurements allow frequent repeated sampling and subjects can simply collect samples themselves, even at home.

It is hypothesized that free lipid-soluble and unconjugated steroids pass through the capillary wall, basement membrane, and acinar cells of the salivary glands along a concentration gradient and enter the saliva [1]. In theory, the concentration of steroid hormones in saliva might be influenced by the salivary flow rate. However, it was reported that cortisol concentrations were similar in unstimulated and stimulated parotid saliva, suggesting that the intracellular excretion route of cortisol is not significantly influenced by the salivary flow rate [1]. Although it has never been investigated so far, it therefore seems likely that other unconjugated steroids are also excreted independent of the salivary flow rate. Clinicians and researchers do often not distinguish between unstimulated and stimulated saliva and little attention has been paid so far to a possible difference between these two types of saliva.

In daily practice several ways of saliva sample collection are used. The most straightforward way is to collect unstimulated whole saliva by waiting until saliva is produced and drooling into a tube. However, some subjects have a very low unstimulated saliva production. Therefore, saliva secretion is often stimulated to produce larger volumes of saliva or decrease the time needed for sample collection. Under stimulated conditions, saliva is mainly produced by the parotid glands, whereas the other salivary glands are mainly responsible for the basal saliva production [2]. Little is known about a possible difference in steroid hormone excretion by the different salivary glands. It is mainly the possible difference between unstimulated whole saliva and stimulated parotid gland
saliva that draws attention, because it could give new information on the excretion of steroids during stimulation.

Saliva secretion can be stimulated in several ways, but is usually stimulated by masticatory action (e.g. chewing chewing gum or Salivette® swabs (Sarstedt, Nümbrecht, Germany)) or by gustatory stimuli (e.g. oral application of citric acid). Salivette® swabs are designed and produced for salivary cortisol measurement (https://www.sarstedt.com; accessed June 10, 2015). However, they are used for the measurement of other hormones in saliva as well [3] although it has been reported that Salivette® swabs can cause problems in the measurement of steroids other than cortisol. Recoveries of testosterone and androstenedione from synthetic and cotton Salivettes® were lower than recoveries from untreated saliva samples and absorption of steroid hormones to the swabs has been suggested as a possible explanation for the observed decrease in testosterone concentration [2;4;5]. On the other hand, other studies found higher testosterone levels when saliva was filtered over cotton Salivette® swabs [6]. In subjects where saliva was collected subsequently without stimulation, with cotton and with synthetic Salivettes®, testosterone levels where higher when saliva was collected with cotton and synthetic swabs [6]. It was suggested that the presence of testosterone, or another compound interfering in the measurement, in cotton swabs causes the elevated testosterone concentrations [7;8]. Due to these conflicting observations, it is still unclear which saliva collection method clinicians and researchers should use for measurement of steroid hormones in saliva.

Therefore, in the present study we investigated whether there is a difference in steroid hormone concentration between unstimulated and stimulated whole saliva; between unstimulated whole saliva and stimulated parotid gland saliva and finally between unstimulated whole saliva and stimulated saliva collected using synthetic and cotton Salivette® swabs, citric acid and chewing gum. The main aim of this study is to provide data to clinicians and researchers about the optimal saliva collection procedure for measurement of several steroid hormones.

Materials and methods

Participants Saliva was collected from healthy volunteers, male and female, aged between 20 and 61 years. Informed written consent was obtained from all participants. Medical conditions and drug use (including oral contraceptives) were recorded. Subjects taking interfering medication such as oral glucocorticoids were excluded. Participants were also excluded if they had obvious oral bleedings. Blood which leaks into the oral mucosa as a result of micro injuries can cause falsely elevated salivary hormone levels [6], as serum steroid hormone concentrations are much higher than those in saliva. To avoid blood contamination in the saliva samples volunteers were requested to avoid dental treatment 48h prior to sample collection, avoid teeth brushing 2h prior to sample collection and avoid eating for at least 1h before sample collection. These criteria were also used in other saliva experiments in literature [9;10].
**Sample Handling** All samples were collected between 9 and 11 am. A total of 5 mL saliva was collected using polypropylene tubes in all experiments. Hormone excretion was calculated using the following formula: Excretion (mol/min) = ([(hormone concentration (mol/L)] x 5 mL)/time needed to collect 5 mL saliva). Salivary pH increases after a light stimulation and increases more with a rising secretion rate [11]. The pH of every sample was measured directly after sample collection using an electronic pH-meter (PHM240, pH/ION Meter, MeterLab, Villeurbanne Cedex, France). The samples were vortexed vigorously and subsequently frozen at -80 °C for at least 24h to break down the mucopolysaccharides in order to allow proper aliquotting for the different hormone analyses. After re-thawing, the samples were centrifuged for 30 minutes at 3000 rpm. The clear supernatant was divided into aliquots and frozen again at -80 °C until analysis. The samples obtained from one subject were analysed in one batch to avoid inter-run variation.

**Hormone measurements** All samples were analysed using Isotope-dilution liquid-chromatography tandem mass spectrometry (ID-LC-MS/MS) methods. For testosterone and androstenedione measurements in saliva, the samples underwent a liquid-liquid extraction using Hexane-diethylether (4:1) after addition of internal standards ([13C₃]-testosterone (Cerilian, Round Rock, Texas) and [13C₃]–androstenedione (Cerilian), before they were injected in a Acquity 2D-UPLC-system which is coupled to a Xevo TQ-S tandem mass spectrometer (Waters Corp., Millford, MA). This method is based on an earlier published LC-MS/MS method for testosterone, androstenedione and DHEA in serum [12]. Intra-assay CVs for testosterone were 22% below 7 pmol/L, 9.6% between 7 and 30 pmol/L and <6.7% above 30 pmol/L (n=362 duplicates). For androstenedione, intra-assay CVs were <6.2% between 50 and 700 pmol/L (n=382 duplicates). Samples with androstenedione concentrations below 50 pmol/L are rare. Inter-assay CVs for testosterone were 12%, 12%, 5.1% and 8% at 13, 34, 159 and 850 pmol/L, (n=30) respectively. For androstenedione inter-assay CVs were 15%, 9.0%, 6.3% and 8% at 14, 45, 153 and 852 pmol/L, respectively (n=28). Cortisol and cortisone were measured after addition of internal standards ([2H₄]-cortisol (Cambridge Isotope Laboratories, Tewksbury, MA) and [2H₈]-cortisone (Toronto Research Chemicals, Toronto, Canada)) using a Symbiosis online solid phase extraction (SPE) system (Spark Holland, Emmen, The Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp.). Intra-assay CVs were <6.4 % for cortisol concentrations above 1 nmol/L and 14% for cortisol concentrations below 1 nmol/L (n=169 duplicates). Intra-assay CVs were <4.7% for cortisone concentrations above 10 nmol/L (n= 169 duplicates). There are very few samples containing less than 10 nmol/L cortisone. Inter-assay CVs were 9.6%, 7.0% and 6.6% for cortisol at 2.1, 11 and 42 nmol/L, respectively, (n=64) and 14%, 8.2% and 8.1% for cortisone at 2.2, 27 and 105 nmol/L, respectively (n=54).

**Experiment 1: Comparison of unstimulated and stimulated whole saliva** In the first experiment steroid hormone concentrations of unstimulated whole saliva and stimulated whole saliva were compared in 10 male and 10 female subjects. The participants were instructed to avoid talking, swallowing and moving their mouth and tongue during unstimulated saliva collection and drool saliva in the tube as soon as it emerged. For every part of the experiment subjects were asked...
to produce 5 mL of saliva. In order to calculate excretion, the time needed to produce 5 mL saliva was noted. Subsequently, the same participants were asked to move their mouth and tongue as much as possible in order to stimulate saliva production and spit this stimulated saliva in a separate tube.

Experiment 2: Comparison of total and parotid gland saliva In the second experiment steroid hormone concentrations of unstimulated whole saliva and stimulated parotid gland saliva were compared in 8 male and 7 female subjects. Unstimulated whole saliva was obtained as described above. Subsequently, parotid saliva was collected with a Lashley cup placed over the orifice of one of the parotid ducts in the mouth (13). Chewing gum was used to stimulate the saliva secretion once the cup was placed.

Experiment 3: Comparison of four methods of salivary stimulation In the third experiment steroid hormone concentrations of unstimulated whole saliva and stimulated whole saliva obtained with four commonly used methods were compared in 10 male and 10 female subjects. Unstimulated whole saliva was obtained as described above. Subsequently, saliva was collected with synthetic (polyethylene) Salivette® swabs and cotton Salivette® swabs (Sarstedt, Nümbrecht, Germany). This was followed by the collection of saliva stimulated by the application of 4% citric acid on the tongue with a cotton swab in one half of the subjects and chewing on a chewing gum (Sportlife mint, sugarfree chewing gum) in the other half of the subjects.

Additional experiments with Salivette® swabs We studied recovery of testosterone and androstenedione from both types of Salivettes® in a separate experiment, to exclude that the differences found were due to the subsequent sample collection in our experiment. Unstimulated whole saliva of ten healthy volunteers (five males and five females) was collected and frozen. After thawing, 2.5 mL saliva was pipetted on a synthetic and a cotton Salivette®. After 10 minutes the swabs were centrifuged for 15 min at 1900 g to recollect the saliva. This saliva was analysed parallel to the untreated saliva. We also filtered 2.5 mL Milli-Q water (Millipore ReferenceA+) over both types of Salivettes® and analysed the filtered water as well as untreated water for testosterone and androstenedione.

Statistical analyses In experiment 1 and 2, pH, steroid hormone concentrations and time needed to collect 5 mL of saliva were analysed using the non-parametric paired Wilcoxon signed-rank test. The non-parametric repeated measures Friedman’s ANOVA was used in experiment 3 to compare measures in unstimulated whole saliva, saliva collected with the use of synthetic and cotton Salivette® swabs, followed by a non-parametric paired Wilcoxon signed-rank as posthoc procedure for pairwise comparisons. Saliva stimulated by citric acid and by chewing gum, was only collected in half of the subjects for each method. For this reason, the salivary hormone concentrations in citric acid and chewing gum stimulated saliva were each separately compared to the unstimulated saliva hormone levels using paired Wilcoxon signed-rank tests. Data are shown as median (range) unless indicated otherwise. P-values < 0.05 were regarded as to reflect statistical significance. All statistical
analyses were performed using SPSS statistical software for Windows version 20 and GraphPad Prism version 6.

**Results**

**Experiment 1: Comparison of unstimulated and stimulated whole saliva** Ten healthy males and ten healthy females aged between 20 and 61 years participated in this experiment. The average time to produce 5 mL of stimulated saliva was shorter than the time to produce a similar volume of unstimulated saliva, 19.6 minutes (range 7.0 - 55.5 minutes) and 10.4 minutes (range 5.0 - 20.5 minutes), for unstimulated and stimulated saliva, respectively (P < 0.01). The pH was higher in stimulated saliva (median pH 7.19) when compared to unstimulated saliva (median pH 6.98) in all subjects (p < 0.01). Hormone concentrations and excretions in unstimulated and stimulated saliva are shown in Figure 1. Testosterone, androstenedione and cortisol concentrations did not differ between unstimulated and stimulated saliva. The median (range) concentrations were 74 (6-301) versus 75 (6-286) pmol/L for testosterone (p = 0.15), 204 (72-516) versus 209 (62-298) pmol/L for androstenedione (p = 0.32) and 3.9 (1.3-19.9) versus 4.2 (1.6-15.7) nmol/L for cortisol (p=0.49), in unstimulated and stimulated saliva, respectively. Cortisone concentrations were on average 16% lower in stimulated saliva (28.6 (15.3-64.3) nmol/L) than in unstimulated saliva (35.0 (18.1-71.9) nmol/L) (p < 0.01). The hormone excretion increased on average 95%, 84%, 104% and 63% for testosterone, androstenedione, cortisol and cortisone concentrations, respectively (p < 0.01 in all cases).

**Experiment 2: Comparison of total and parotid gland saliva** Eight healthy males and seven healthy females aged between 20 and 61 years participated in this experiment. The pH was higher in stimulated parotid saliva (median pH 7.33) when compared to unstimulated whole saliva (median pH 6.95) (p < 0.01). Most of the subjects had a higher pH in stimulated parotid saliva, however three of the subjects had a slightly lower pH in parotid saliva. Hormone concentrations in unstimulated whole saliva and parotid saliva are shown in Figure 2. Median testosterone, androstenedione, cortisol and cortisone concentrations were lower in stimulated parotid saliva when compared to unstimulated whole saliva (87 (11-312) versus 113 (14-484) pmol/L (mean difference was 51 %; p < 0.01). for testosterone, 227 (91-370) versus 230 (136-483) pmol/L for androstenedione (mean difference was 26% (p = 0.02); 11.0 (3.0-77.7) versus 16.9 (1.8-109) nmol/L for cortisol (mean difference was 66%; p = 0.02) and 104 (32.7-238) versus 137 (19.8-326) nmol/L for cortisone (mean difference was 49%; p < 0.01), in stimulated parotid and unstimulated whole saliva, respectively). Testosterone, cortisol and cortisone excretions were higher in parotid saliva when compared to unstimulated whole saliva: mean differences were 33% (p = 0.04), 9% (p = 0.04) and 23% (p < 0.01) for testosterone, cortisol and cortisone concentrations, respectively. Androstenedione excretion was not significantly different between parotid saliva and unstimulated whole saliva (p = 0.18). Data not shown.
Figure 1: Concentration (a) and excretion (b) of testosterone (I), androstenedione (II), cortisol (III) and cortisone (IV) in unstimulated and stimulated saliva (n=20). ( * = P < 0.05).
Figure 2: Concentrations of testosterone (I) androstenedione (II), cortisol (III) and cortisone (IV) in unstimulated whole and stimulated parotid saliva. (n=15) (* = P < 0.05).
Figure 3: Concentrations of testosterone (I), androstenedione (II), cortisol (III) and cortisone (IV) in unstimulated and saliva stimulated by synthetic Salivettes, cotton Salivettes, citric acid and chewing gum (n=20). (★ = P < 0.05).
Experiment 3: Comparison of four methods of salivary stimulation

Ten healthy males and ten healthy females aged between 20 and 55 years participated in this experiment. Hormone concentrations in unstimulated and several types of stimulated saliva are shown in Table 1, 2 and 3 as well as Figure 3. When compared to unstimulated saliva, median testosterone concentrations were much lower in saliva collected with synthetic Salivette® swabs. The mean difference was 58% (p < 0.01). Saliva collected with cotton Salivette® swabs had 217% (p < 0.01) higher testosterone concentrations than unstimulated saliva. Chewing gum stimulated saliva had on average 55% lower testosterone concentrations than unstimulated saliva (p < 0.01). Citric acid stimulated saliva had equal testosterone concentrations as unstimulated saliva (p = 0.93). Androstenedione concentrations were 41% (p < 0.01) lower in saliva collected with synthetic Salivette® swabs when compared to unstimulated saliva. In contrast, androstenedione concentrations were 46% (p < 0.02) higher in saliva collected with cotton Salivette® swabs when compared to unstimulated saliva. Citric acid stimulated saliva as well as chewing gum stimulated saliva had equal androstenedione concentrations compared to unstimulated saliva (p = 0.59 and p = 0.22, for citric acid and chewing gum stimulation, respectively). One subject outlied the others severely showing much higher androstenedione concentrations in all stimulations. Saliva collected with synthetic Salivettes® tended to have lower cortisol concentrations as unstimulated saliva and saliva collected with cotton Salivettes® tended to have higher cortisol concentrations. However, this decrease was not significant in the Friedman’s ANOVA (p = 0.052). Cortisol concentrations tended to be higher in saliva stimulated by citric acid (p = 0.056) and stimulation by chewing gum decreased cortisol concentration on average 16% when compared to unstimulated saliva (p = 0.04). Saliva collected with synthetic and cotton Salivettes® had lower cortisone levels when compared to unstimulated saliva. Mean decrease was 25% (p < 0.01) and 34% (p < 0.01) for saliva collected with synthetic and cotton Salivette® swabs, respectively. Also, saliva stimulated by citric acid and by chewing gum had lower cortisone concentrations than unstimulated saliva. The mean decrease was 35% (p < 0.01) and 25% (p < 0.01) for citric acid and chewing gum stimulation, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated saliva</th>
<th>Stimulated saliva</th>
<th>Mean change (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testosterone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Synthetic Salivette</td>
<td>14 (1.5-274) pmol/L</td>
<td>-58%</td>
<td>p&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Cotton Salivette</td>
<td>82 (5-500) pmol/L</td>
<td>217%</td>
<td>p&lt;0.01</td>
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<tr>
<td><strong>Androstenedione</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthetic Salivette</td>
<td>80 (32-259) pmol/L</td>
<td>-41%</td>
<td>p&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Cotton Salivette</td>
<td>220 (127-512) pmol/L</td>
<td>46%</td>
<td>p&lt;0.02</td>
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<tr>
<td><strong>Cortisol</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Concentration</td>
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<td></td>
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<tr>
<td>Synthetic Salivette</td>
<td>5.4 (1.4-27.0) nmol/L</td>
<td>-8%</td>
<td>p=0.052</td>
<td></td>
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<tr>
<td></td>
<td>Cotton Salivette</td>
<td>Synthetic Salivette</td>
<td>Cotton Salivette</td>
<td>3%</td>
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<tr>
<td>Cortisone</td>
<td>5.7 (1.1-23.1) nmol/L</td>
<td>22.7 (8.4-49.3) nmol/L</td>
<td>21.1 (5.5-45.8) nmol/L</td>
<td>-25%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-34%</td>
</tr>
</tbody>
</table>

Table 1: Concentrations of testosterone, androstenedione, cortisol and cortisone in unstimulated saliva and saliva stimulated by synthetic and cotton Salivettes® as well as the mean differences between unstimulated and each of the stimulations. Concentrations of testosterone and androstenedione are in pmol/L; concentrations of cortisol and cortisone are in nmol/L (n=20). All concentrations are given as median (range).
### Table 2: Concentrations of testosterone, androstenedione, cortisol and cortisone in unstimulated saliva and saliva stimulated by citric acid as well as the median differences between unstimulated and citric acid stimulation stimulation. Concentrations of testosterone and androstenedione are in pmol/L; concentrations of cortisol and cortisone are in nmol/L (n=10). All concentrations are given as median (range).

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated</th>
<th>Citric acid</th>
<th>Mean change (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>159 (8-475) pmol/L</td>
<td>42 (4-515) pmol/L</td>
<td>18%</td>
<td>p=0.93</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>189 (115-655) pmol/L</td>
<td>86 (107-553) pmol/L</td>
<td>-3%</td>
<td>p=0.59</td>
</tr>
<tr>
<td>Cortisol</td>
<td>9.0 (1.2-25.8) nmol/L</td>
<td>7.1 (1.3-21.9) nmol/L</td>
<td>12%</td>
<td>p=0.056</td>
</tr>
<tr>
<td>Cortisone</td>
<td>37.7 (11.2-72.3) nmol/L</td>
<td>23.1 (7.5-38.5 nmol/L)</td>
<td>-35%</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

### Table 3: Concentrations of testosterone, androstenedione, cortisol and cortisone in unstimulated saliva and saliva stimulated by chewing gum as well as the median differences between unstimulated and chewing gum stimulation. Concentrations of testosterone and androstenedione are in pmol/L; concentrations of cortisol and cortisone are in nmol/L (n=10). All concentrations are given as median (range).

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated</th>
<th>Chewing gum</th>
<th>Mean change (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>18 (4-198) pmol/L</td>
<td>23 (7-302) pmol/L</td>
<td>55%</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>143 (55-449) pmol/L</td>
<td>192 (84-392) pmol/L</td>
<td>49%</td>
<td>p=0.22</td>
</tr>
<tr>
<td>Cortisol</td>
<td>4.5 (1.5-10.4) nmol/L</td>
<td>5.5 (1.8-12.2) nmol/L</td>
<td>16%</td>
<td>p=0.04</td>
</tr>
<tr>
<td>Cortisone</td>
<td>28.6 (12.9-53.6) nmol/L</td>
<td>2.0 (10.5-41.9) nmol/L</td>
<td>25%</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

**Additional experiments with Salivette® swabs** Testosterone and androstenedione concentrations measured in saliva filtered over synthetic swabs were on average 28% (range 19-75%; mean difference 30 pmol/L (range 4-51 pmol/L)) and 23% (range 15-29%; mean difference 63 pmol/L (range 20-131 pmol/L)) lower than untreated saliva, respectively. Testosterone and androstenedione concentrations measured in saliva treated with cotton swabs were on average 242% (range 0-686%; mean difference 31 pmol/L (range -1 – 50 pmol/L)) and 40% (range -11 – 186%; mean difference 52 pmol/L (range -78 – 144 pmol/L)) higher than untreated saliva, respectively. Milli-Q-Water filtered over synthetic swabs contained less than 10 pmol/L testosterone and androstenedione, whereas water filtered over cotton swabs contained on average 71 pmol/L (range: 60-90 pmol/L) testosterone and 136 pmol/L (range: 120-160 pmol/L) androstenedione. The water on itself did not contain these hormones.

**Discussion**

In the present study we compared steroid hormone concentrations in unstimulated and stimulated whole saliva, as well as in whole and parotid saliva. In addition, we investigated whether commonly used saliva collection methods interfere with steroid hormone measurements.
Unstimulated and stimulated whole saliva had similar concentrations of testosterone, androstenedione and cortisol. This confirms the suggestion of Vining et al. [1] that unconjugated steroids like testosterone and androstenedione behave similar to cortisol and that their excretion is independent of the salivary flow rate. This is most probably due to the remarkable increase in hormone excretion which accompanies the increase in saliva production seen during stimulation. Although cortisone excretion increased, this increase was not enough to maintain a stable cortisone concentration. This observation, not previously reported, might be due to a rate limitation of the enzymatic conversion of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in the salivary glands [14]. As opposed to the results in whole stimulated saliva, we showed that testosterone, androstenedione, cortisol and cortisone concentrations are lower in stimulated parotid gland saliva than in unstimulated whole saliva. It is unclear what has caused this difference, but, based on the data showed by our first experiment and the study of Vining et al. [1], it seems unlikely that the stimulation per se is the explanation for this difference in testosterone, androstenedione and cortisol concentration. For cortisone, however, the lower levels in stimulated parotid saliva, might also be caused by a failure to increase cortisone excretion enough to maintain stable concentrations upon stimulation of the saliva production, as was observed in the first experiment. By our knowledge, there are no other data on steroid hormone concentration in saliva of the different glands available. Most probably, the observed differences in steroid concentration between total and parotid saliva is caused by different steroid excretion and/or metabolism in the different salivary glands. The steroid hormone excretion of the parotid gland might be lower than the excretion of other salivary glands. This might be explained by the observation that parotid saliva contains different proteins than other types of saliva [15] and might therefore absorb less easily steroid hormones and by this influencing the diffusion equilibrium in the salivary gland.

We observed remarkable effects of the type of saliva collection methods on steroid hormone concentrations. As expected from earlier observations and data of the manufacturer, cortisol levels were not influenced by stimulation with Salivette® swabs. On the contrary, testosterone and androstenedione concentrations were dramatically lower when synthetic Salivettes® were used. This observation is in line with previous studies [2;4;5], where low recoveries of steroid hormones from these swabs were found. We proved that this phenomenon also occurs when synthetic Salivettes® were used for sample collection. The loss of testosterone and androstenedione in saliva filtered over synthetic Salivettes® strongly suggests that these synthetic swabs absorb testosterone and androstenedione, as previously suggested [5-7], and should be avoided when these hormones are to be measured. It is probably due to the chemical properties of testosterone and androstenedione, that these behave so different from cortisol. It is not clear why Granger et al. [6] reported higher testosterone concentrations when using synthetic Salivettes® for saliva collection, but cross reaction or other problems in the immunoassay used in Granger’s study might be responsible for the conflicting results. One of the strengths of our study is the use of ID-LC-MS/MS methods for the steroid measurement. ID-LC-MS/MS is nowadays recommended for steroid hormone analysis, mainly because of its high specificity and lower variation [16]. Sensitive and accurate ID-LC-MS/MS methods
for salivary steroids might distinguish between biological effects and method artefacts as well as interferences from collection methods.

Opposite to the lower androgen concentrations upon synthetic Salivettes® use, the use of cotton Salivettes® caused dramatically higher testosterone and androstenedione concentrations. This is in line with Granger et al. [6], who showed that the use of cotton swabs increased testosterone levels. It has been suggested that cotton contains plant hormones that might diffuse into the saliva and interfere with the steroid measurements [7;8]. The increase in testosterone and androstenedione concentrations in saliva filtered over cotton swabs as well as the detection of both hormones in water filtered over cotton swabs, strongly support this suggestion that testosterone and androstenedione, or at least compounds that behave exactly the same as these steroids, chromatographically and in the mass spectrometer, are present in cotton Salivette® swabs. In short, our data show that both types of Salivette® swabs are not suitable for salivary testosterone and androstenedione measurements.

Testosterone and cortisol levels were lower in saliva stimulated by chewing gum. Probably, this latter phenomenon was caused by absorption to the chewing gum, which should thus being avoided in salivary steroid analysis. Citric acid did not influence testosterone, androstenedione and cortisol concentrations. However, citric acid should be used carefully, as acidification of the sample may interfere with immunoassays [17]. As was seen in all experiments, cortisol concentrations are lower in stimulated saliva, independent of the type of stimulation. It is unclear, whether this is a consequence of stimulation alone or also influenced by the different stimulators used in the third experiment. The decrease in cortisol concentration was more pronounced in the stimulations in the third when compared to the first experiment. However, in this experiment setup it is not possible to distinguish between an effect of extra stimulation by the different stimulators or a direct effect, such as absorption to the swabs or chewing gum, of the stimulators on cortisol concentrations. As mentioned above, the decrease seen in cortisol concentration might be due to a rate limitation of the enzymatic conversion of cortisol in cortisone by 11β-HSD2 in the salivary glands [14].

In conclusion, stimulation of saliva secretion per se does not change testosterone, androstenedione and cortisol concentrations, but decreases cortisone levels. Salivette® swabs should be avoided for salivary steroid hormone measurements, with the exception of cortisol. Based on the present data unstimulated saliva collection in a simple polypropylene tube is the best choice for all salivary steroid hormone measurements.

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References


