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Potential of saliva steroid profiling for the detection of endogenous steroid abuse: Reference thresholds for oral fluid steroid concentrations and ratios

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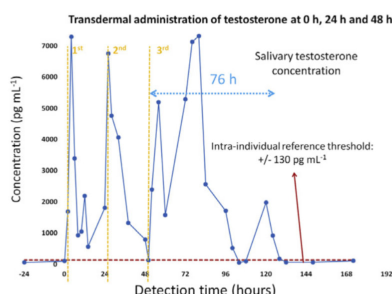
HIGHLIGHTS

- A method for oral fluid doping controls is proposed.
- Reference thresholds for saliva steroid profile parameters are presented.
- Steroid profile ratios are superior for longitudinal monitoring.
- Saliva outperforms urine for detecting a transdermal testosterone administration.
- Isotope ratio mass spectrometry is no longer required for confirmation.

GRAPHICAL ABSTRACT



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ABSTRACT

Urine and blood samples are the primary matrices for the detection of exogenous substances in doping control and toxicology. Although these matrices are, in general, very suitable for a wide range of substances, they do show some issues in particular cases. Here, alternative matrices may provide an answer.

In this work, a quantitative method for steroid profiling (5 endogenous steroids and their ratios) in oral fluid was developed and validated. In total, 826 saliva samples were analyzed, and inter-individual reference population thresholds for saliva steroid profile parameters were set up. Alterations of this steroid profile after administration of naturally occurring anabolic androgenic steroids (e.g. testosterone (T) or dehydroepiandrosterone (DHEA)) were investigated. In addition, intra-individual short and long-term natural fluctuations were investigated. For longitudinal monitoring in oral fluid, steroid profile ratios (e.g., T/DHEA) were superior to absolute concentrations due to lower susceptibility towards the diurnal pattern.

For the detection of a transdermal application of T, the salivary parameter T/DHEA proved to have the highest sensitivity. In contrast with the current screening procedures in urine, there is no need for an additional expensive and time-consuming isotope ratio mass spectrometry confirmation procedure to unequivocally attribute the elevated parameter to an exogenous origin.

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1. Introduction

In diagnostics, saliva is regarded as a promising medium for the detection and prediction of disease progression [1]. Especially the stress-free, non-invasive nature of saliva and its fast collection make it a valuable alternative to urine and/or blood. Previous papers reported that salivary testosterone (T) concentrations correlate well with serum free T concentrations and suggested that saliva could be an equally well-performing matrix [2–8]. A recent work that performs a critical evaluation of salivary T and its relation to serum T concluded that passive drooling is the most appropriate collection method [9].

Saliva testing is increasingly popular as an alternative matrix to screen for drugs of abuse in clinical toxicology, workplace testing, emergency room testing, etc. [10] This led to the introduction of guidelines for workplace oral fluid testing by the Substance Abuse and Mental Health Services Administration and the European Union's Driving under the influence of Drugs, Alcohol and Medicines program [11,12].

Doping control mainly relies on testing urine (and to a lesser extent blood) samples. These matrices are authorized by the World Anti-Doping Agency (WADA) and are collected and transferred to accredited doping control laboratories under standardized conditions. They have proven their usefulness and importance and provide answers and solutions to many of the confronted issues concerning the detection of doping substances. However, some issues remain, and alternative complementary matrices can provide an answer. For example, transdermal administration of T is difficult to detect by analyzing urine and is much easier to detect in saliva [13–15].

Research on saliva as a matrix for sports doping control purposes has been limited due to the very low concentrations of target analytes in oral fluid [16–18]. For example, endogenous anabolic androgenic steroids (AAS) are present in saliva in the pg mL^{-1} range, whereas they reach concentrations in the order of ng mL^{-1} range in urine [19–21]. Due to the evolution in analytical instrumentation, these detection limits can now be reached [22–26]. This creates an opportunity to use saliva as a collection matrix in doping control. Nevertheless, prior to its routine use, much research and data are still required to confirm that saliva not only offers opportunities but also has an added value and provides equally valid results [17,27].

For an effective detection of doping abuse using endogenous AAS (e.g., T and dehydroepiandrosterone (DHEA)), reference population thresholds need to be defined to be able to differentiate between normal endogenous steroid concentrations and elevated concentrations caused by an AAS administration. Similarly, statistically-based thresholds for selected relevant endogenous steroid concentrations and ratios, rather than for a single steroid, were set up for urine in the eighties and nineties [21,28–31]. At a later stage, due to the improvements in instrumental sensitivity, reference ranges for minor endogenous metabolites were added [20,32–34]. The combination of these steroid concentrations and ratios is known as the steroid profile and is crucial for enhanced detection of misuse of a range of steroid preparations (dihydrotestosterone (DHT), DHEA, T, etc.) and formulations. In addition to the inter-individual reference thresholds, the short and long-term concentration fluctuations in saliva for an individual are of importance if an Athlete Biological Passport (ABP) with intra-individual thresholds is to be set up, in parallel with the steroid module in the ABP for urine [35].

This study aims at setting up a saliva steroid profile (with corresponding reference ranges) and evaluating the intra-individual longitudinal stability of these concentrations and ratios (e.g., intra-individual reference ranges). These data are currently missing in the literature and are required to start conducting routine doping control analyses using saliva.

2. Materials and methods

2.1. Reagents and reference standards

Ethanethiol was bought from Acros (Geel, Belgium). N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from Chem. Fabrik Karl Bucher (Waldstedt, Germany); ammonium iodide (NH_4I), dodecane, androstenedione (ADION), T, cortisone and cortisol were obtained from Sigma Aldrich (Bornem, Belgium). DHEA originated from Serva (Heidelberg, Germany). Testosterone-d3 (T-d3) was bought from the National Measurement Institute (Pymble, Australia). Cortisol-d4 and cortisone-d8 originated from Cerilliant (Texas, United States). Acetonitrile and methyl *tert*-butyl ether were from Biosolve (Valkenswaard, The Netherlands). β-Glucuronidase from *E. coli* K12 was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Sodium hydrogen carbonate ($NaHCO_3$) and diethyl ether were from Fisher Scientific (Loughborough, UK). Potassium carbonate (K_2CO_3), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium acetate (NaOAc) and sodium sulfate (Na_2SO_4) were obtained from Merck (Darmstadt, Germany).

Phosphate buffer (pH 7) was prepared by dissolving 7.1 g of $Na_2HPO_4 \cdot 2H_2O$ and 1.4 g of $NaH_2PO_4 \cdot H_2O$ in 100 mL of water. Carbonate buffer (pH 9.5) was prepared by dissolving 135 g of K_2CO_3 and 111 g of $NaHCO_3$ in 900 mL of double-distilled water.

2.2. Inter- and intra-reference population and administration study

Approval from the ethical committee of Ghent University Hospital (EC/2015/1316) was obtained, and the studies were performed according to the Helsinki declaration. Written consent was obtained allowing the use of the samples for research purposes.

Saliva samples were collected via passive drooling from employees of Ghent University during their yearly medical check at the Ghent University Hospital. The reference population consisted of 387 males and 439 females ranging from 18 to 74 years old. There was a high representation of young individuals (74% < 33 years). This population closely resembles the population that doping control laboratories are dealing with (i.e., competitive athletes). Women taking hormonal oral contraceptives were included in the population because this is also the case in routine doping control samples where there is no mandatory declaration on doping control forms for the use of oral contraceptives. Additionally, including this population avoids possible biases that may result from discarding this substantial proportion of women. Due to differences in endocrinology, men and women were studied separately. The number of samples from males and females, included in the database, was sufficient to ensure statistically reliable inter-individual reference levels for each threshold limit for both sexes.

For the short term intra-individual variability study, 6 males and 7 females participated. Here, a saliva sample was collected every morning, noon and evening for 7 consecutive days. For the long term intra-individual variability study, 5 males and 5 females participated. Here, 10 saliva samples were gathered over a time period of 6 months.

T gel (Testim 50 mg/5 g) was applied to the chest and upper arms of one male subject (29 years, 81 kg, genotype ins/del) three times, with intervals of 24 h. Blank urine and saliva samples were collected 24 h before the first administration and immediately prior to the first administration (at 0 h). Then, urine and saliva samples were collected for nine days. T gel was administered immediately after the collection of the samples at 0 h, 24 h and 48 h.

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