Document Number:	TL21	Version Number:	2.0
Written by: Reviewed by:	*WADA* Science *WADA* Laboratory Expert Group	Approved by:	*WADA* Executive Committee
Date:	21 December 2020	Effective Date:	1 January 2021

IN SITU FORMATION OF 4-ANDROSTENE-3,6,17-TRIONE (6-OXO) AND *METABOLITES*

1.0 Introduction

WADA wishes to draw the attention of the Laboratories to the possible detection of **6-Oxo-androstenedione (6-oxo)** and *Metabolites* in urine *Samples* resulting from the *in situ* transformation of **DHEA**.

It has been observed that microbial contamination may induce modifications in the structure of some endogenous steroids (*e.g.,* DHEA) by oxidoreductive and other reactions, leading to the formation of hydroxylated and oxidized derivatives, which may hamper the interpretation of results (Figure 1) [1,2]. In additon, some steroids which are normally excreted as glucuronide conjugates [*e.g.* 6α-hydroxy *Metabolites* of 6-oxo such as **6α-hydroxyandrostenedione (6α-OH-AD)** and **6α-hydroxytestosterone (6α-OH-T)**] might be detected as aglycons because of bacteria-mediated hydrolysis [3,4].

Figure 1 illustrates the possibility of *in situ* biotransformation of endogenous DHEA into the prohibited aromatase inhibitor 6-oxo, which would be detected in a urine *Sample* without its major *Metabolite* 6α-OH-AD [2,5].

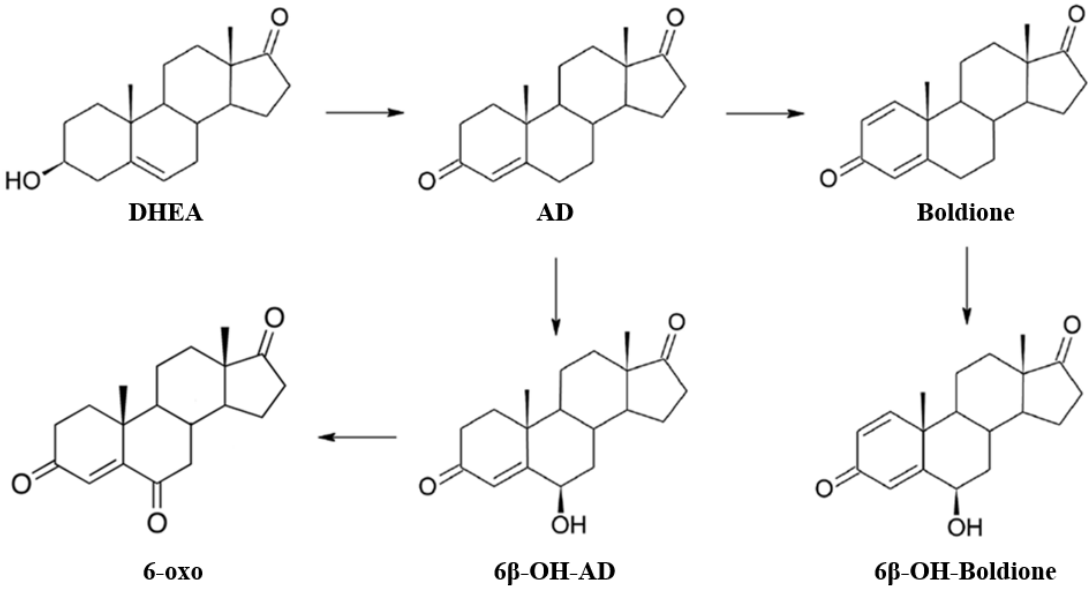

Figure 1. DHEA oxidation followed by isomerization of the double bond, formation of androst-4-ene-3,17-dione (AD), boldione and the respective 6β-OH-derivatives. The oxidation of the hydroxyl group at the C6-β position leads to the formation of 4-androstene-3,6,17-trione (6-oxo) [2,5].

Therefore, Laboratories should be cautious when detecting 6-oxo in a urine *Sample* in the absence of the glucuronidated form of its major 6α-hydroxy *Metabolite* 6α-OH-AD [6,7,8]. Further, since the isomer

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6β-OH-AD may also be formed due to *in situ* biotransformation of DHEA or *via* light-induced auto-oxidation of the corresponding trimethylsilyl 3,5-dienol ethers [9], the Confirmation Procedure (CP) should be carefully reviewed to avoid an incorrect interpretation which may lead to an erroneous conclusion.

It is noted that 6α-OH-AD may be formed endogenously and detected at low concentrations as a minor *Metabolite* in urine *Samples* (typically < 5 ng/mL, although endogenous concentrations > 5 ng/mL have been reported) [10]. However, 6α-OH-AD concentration may also increase as a result of microbial activity and, therefore, 6-oxo may be detected in a *Sample* as a by-product of the enzymatic formation of 6α-OH-AD from either endogenous and/or microbial transformation origin. Therefore, the Laboratory shall perform GC/C/IRMS analysis (depending on Laboratory's analytical capacity, which may require the subcontracting of the analysis to another Laboratory) when the concentration of 6α-OH-AD is greater than (>) 10 ng/mL and there are no signs of extensive *Sample* degradation, even if 6-oxo is present in the *Sample* (see TD IRMS [11]).

2.0 Analysis and Reporting Requirements

Laboratories shall implement the following course of actions before reporting an *Adverse Analytical Finding* (*AAF*) for 6-oxo:

1. Perform a CP using an extraction method [(*e.g.,* Solid Phase Extraction (SPE)] prior to the enzymatic hydrolysis step in order to avoid inducing the *in situ* formation of 6-oxo by the enzymatic activity of microbes already present in the *Sample*;

   [Comment: However, if the side products have already been formed prior to the enzymatic hydrolysis, SPE will have no impact.]

2. Evaluate the overall pattern of *Metabolites* in the *Sample*: 6α-OH-AD shall be detected, and the corresponding 6β-isomers should not be observed in the urine *Sample*. To verify this, the CP should include a step which preserves the stereochemical integrity at C6 (*e.g.*, by derivatization using MSTFA, potassium acetate and imidazole [6,9] or by using a reverse-phase column for LC separation [7]);

   [Comment: 6α-OH-AD can also be found in a Sample as a Metabolite of exogenous synthetic steroids, which are halogenated at the C6-position, e.g. 6α-bromoandrostenedione.]

3. During the CP, verify the conjugated state of 6-oxo *Metabolites*;

4. Evaluate the carbon isotope ratio of 6α-OH-AD by GC/C/IRMS if the concentration of 6α-OH-AD in the *Sample* is greater than (>) 10 ng/mL (SG-adjusted, if needed [11]) and there are no signs of extensive *Sample* degradation (see TD EAAS [12]).

When reviewing an analytical finding for 6-oxo, Laboratories should consider the following reporting recommendations (see also Figure 2).

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- The finding shall be reported as a Negative Finding if:

  o 6-oxo is detected in conjunction with 6β-OH-AD only; and/or

  o 6-oxo glucuronide *Metabolites* are not detected; and/or

  o 6α-OH-AD is identified (in the presence or absence of 6-oxo) at a concentration equal to or less than (≤) 10 ng/mL; and/or

  o 6α-OH-AD is identified (in the presence or absence of 6-oxo) at a concentration greater than (>) 10 ng/mL (SG-adjusted, if needed [11]), but the *Sample* shows signs of extensive degradation;

  o 6α-OH-AD is identified (in the presence or absence of 6-oxo) at a concentration greater than (>) 10 ng/mL (SG-adjusted, if needed [11]), with no signs of extensive *Sample* degradation, and the GC/C/IRMS analysis demonstrates an endogenous origin of 6α-OH-AD.

- The finding shall be reported as an *Atypical Finding* (*ATF*) if:

  o 6α-OH-AD is identified (in the presence or absence of 6-oxo) at a concentration greater than (>) 10 ng/mL (SG-adjusted, if needed [11]), with no signs of extensive *Sample* degradation, and the GC/C/IRMS analysis is inconclusive or cannot be performed;

  o If an *ATF* is reported, the Laboratory shall include a comment in the *ADAMS* Test Report recommending the *Testing* Authority to conduct at least one (1) follow-up no-notice test on the *Athlete* within a reasonable time frame (*e.g.* within 2 weeks).

- The finding shall be reported as an *AAF* if:

  o 6α-OH-AD is identified (in the presence or absence of 6-oxo) at a concentration greater than (>) 10 ng/mL (SG-adjusted, if needed [11]), with no signs of extensive *Sample* degradation, and the GC/C/IRMS analysis demonstrates an exogenous origin of 6α-OH-AD;

  o When the results indicate an *AAF* for 6-oxo and/or 6α-OH-AD, it is recommended that the Laboratory seeks a second opinion, in writing, from another Laboratory before reporting the *AAF*. The second opinion shall be recorded in the Laboratory Documentation Package.

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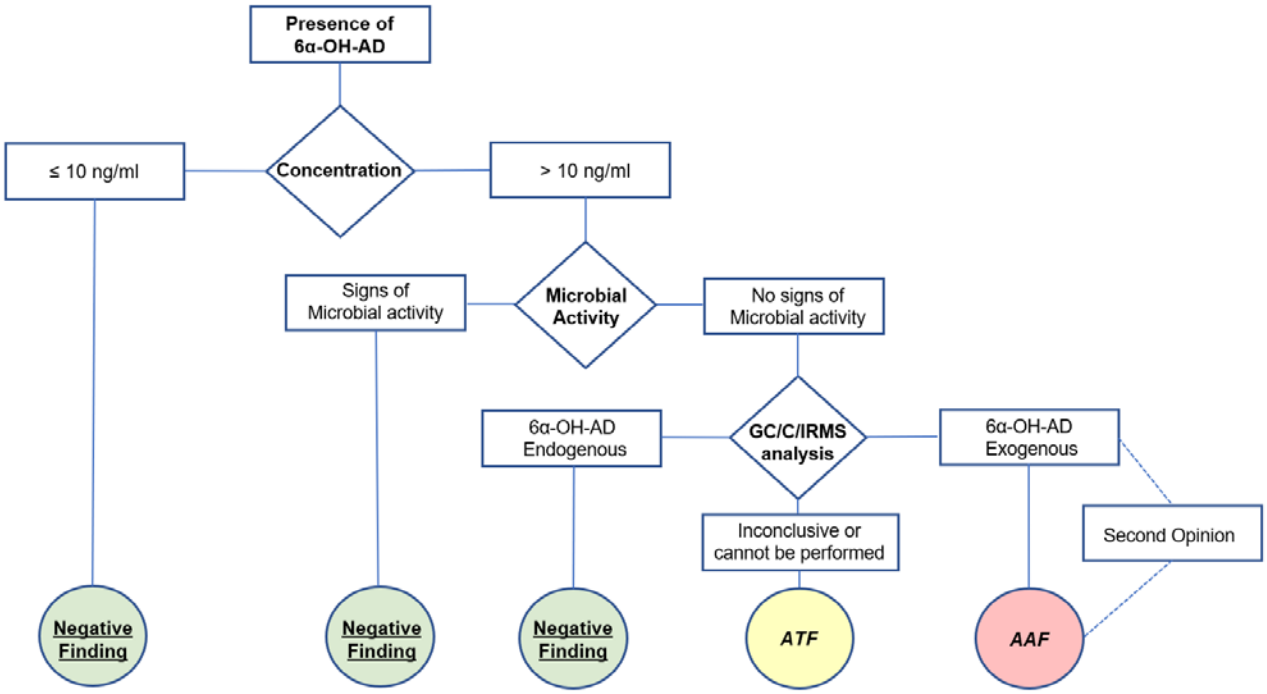

Figure 2. Summary of 6α-OH-AD evaluation.

3.0 References

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[11] *WADA Technical Document* TD IRMS (current version): Detection of Synthetic Forms of *Prohibited* Substances by GC/C/IRMS.

[12] *WADA Technical Document* TD EAAS (current version): Measurement and Reporting of Endogenous Anabolic Androgenous Steroid (EAAS) *Markers* of the Urinary Steroid Profile.

[Current versions of WADA Technical Documents may be found at https://www.wada-ama.org/en/what-we-do/science-medical/laboratories]