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Written by: Reviewed by:	*WADA* Science *WADA* Laboratory Expert Group	Approved by:	*WADA* Executive Committee
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IN SITU FORMATION OF TESTOLACTONE

1.0 Introduction

WADA wishes to draw the attention of the Laboratories to the possible detection of **Testolactone** in urine *Samples* resulting from the *in situ* transformation of **Dehydroepiandrosterone (DHEA)**.

It is noted that minor metabolic pathways from less common microbial contaminations may induce modifications in the structure of the endogenous steroids. For example, filamentous fungus species are able to transform DHEA into different derivatives by 1,2-dehydrogenation and hydroxylation at C-6 or by Baeyer-Villiger (BV) lactonization of the D-ring [1].

Therefore, DHEA may undergo isomerization and oxidation to form **Androst-4-ene-3,17-dione (AD)** [2], which subsequently undergoes BV oxidation into testolactone (Figure 1.1). DHEA may also be transformed directly into its corresponding **3β-hydroxylactone** (Figure 1.2) [1].

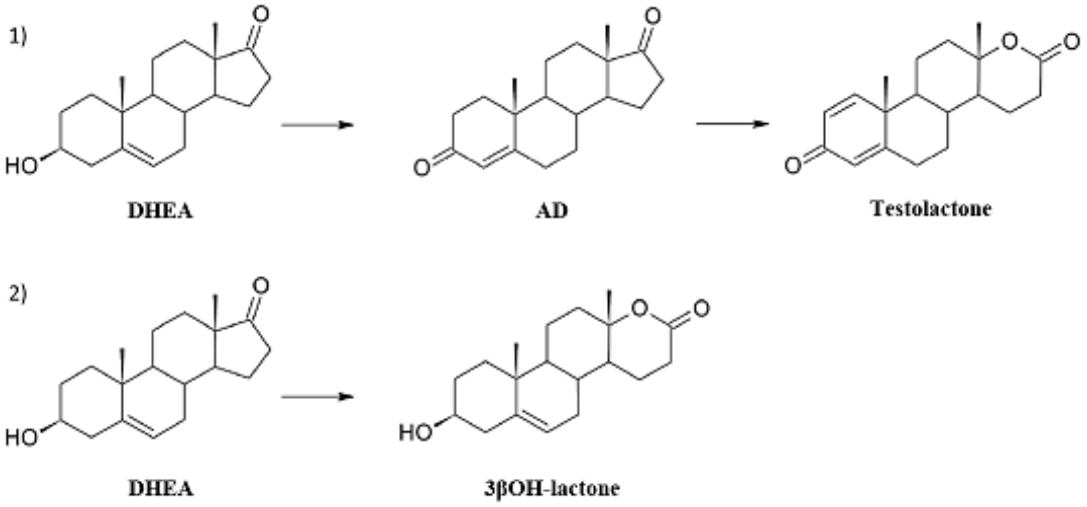

Figure 1. Two possible transformation pathways of DHEA by Baeyer-Villiger (BV) oxidation of the D-ring are presented: 1) At first, isomerization and oxidation of the 3-hydroxy-5-ene moiety into 3-oxo-4-ene occurs, and then BV oxidation of the D-ring takes place to form testolactone; 2) a direct transformation of DHEA into its corresponding 3β-hydroxylactone.

Since many microorganisms can produce an overabundance of steroid-transforming enzymes, which lead, in turn, to multiple transformations [3], it is possible that these two reactions depicted in Figure 1 may occur simultaneously, so that the 3β-hydroxylactone, AD and testolactone are formed in a urine *Sample* during a microbial steroid degradation process. Thus, a urine *Sample* may be found to contain the prohibited aromatase inhibitor testolactone (due to the *in situ* biotransformation of DHEA) in the absence of its major *Metabolites* **4,5-dihydrotestolactone** and **1,2,4,5-tetrahydrotestolactone**, but in

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the presence of a 3β-hydroxylactone compound, which is not a testolactone *Metabolite* but an isobar of another testolactone *Metabolite*: **D-homo-17α-oxa-3-hydroxy-5β-androst-1-ene-17-one** [4].

2.0 Analysis and Reporting Requirements

Before reporting a result as an *Adverse Analytical Finding* (*AAF*) for testolactone, Laboratories shall evaluate whether the finding is the result of the *in situ* transformation of DHEA.

1. Perform a Confirmation Procedure (CP) using an extraction method [*e.g.,* Solid Phase Extraction (SPE)] prior to the enzymatic hydrolysis in order to avoid inducing the *in situ* formation of testolactone 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 testolactone *Metabolites*: at least one (1) of these two (2) major *Metabolites,* 4,5-dihydrotestolactone or 1,2,4,5-tetrahydrotestolactone, shall be confirmed.

3. 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.

3.0 References

[1] Kozłowska E., *et al*. Biotranformation of dehydroepiandrosterone (DHEA) by environmental strains of filamentous fungi. *RSC adv*. **7**(50): 31493-31501, 2017.

[2] *WADA* Technical Letter TL18. *In situ* Formation of 4-Androstene-3,6,17-trione (6-oxo) and *Metabolites*.

[3] Charney W., *et al*. Microbial transformations of steroids: a handbook. Academic Press, 2014.

[4] Van Eenoo P., and Delbeke F.T. Metabolism and excretion of anabolic steroids in doping control - new steroids and new insights. *J Steroid Biochem Mol Biol*. **101**(4-5): 161-178, 2006.

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