

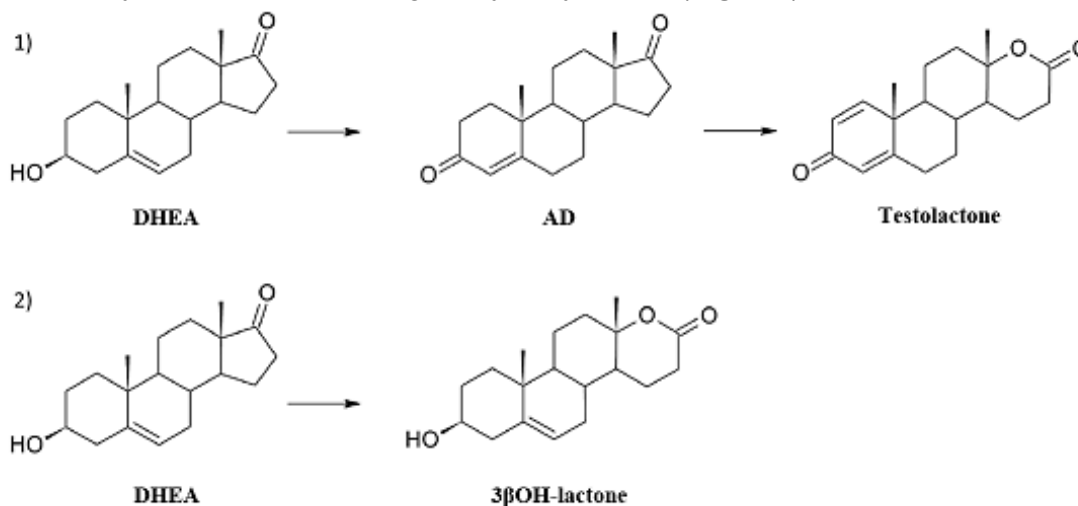
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Written by:	WADA LabEG	Approved by:	WADA Executive Committee
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### **IN SITU FORMATION OF TESTOLACTONE**

The *World Anti-Doping Agency* wishes to draw the attention of the Laboratories to the following analytical issue that may affect Laboratory operations. This pertains, in particular, to the possible detection of testolactone in urine *Samples* resulting from *in situ* transformation of 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 dehydroepiandrosterone (DHEA) into different derivatives by 1,2-dehydrogenation and hydroxylation at C-6 or by Baeyer-Villiger (BV) lactonization of the D-ring <sup>1</sup>.

Therefore, DHEA may undergo isomerization and oxidation to form androst-4-ene-3,17-dione (AD)<sup>2</sup>, which subsequently undergoes BV oxidation into testolactone (**Fig 1.1**). DHEA may also be transformed directly into its corresponding 3 $\beta$ -hydroxylactone (**Fig. 1.2**) <sup>1</sup>.



**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 3 $\beta$ -hydroxylactone.

Since many microorganisms can produce an overabundance of steroid-transforming enzymes, which lead, in turn, to multiple transformations <sup>3</sup>, it is possible that these two reactions depicted in **Fig. 1** may occur simultaneously, so that the 3 $\beta$ -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 the presence of a 3 $\beta$ -hydroxylactone compound, which is not a testolactone *Metabolite* but an isobar of another testolactone *Metabolite*: D-homo-17 $\alpha$ -oxa-3-hydroxy-5 $\beta$ -androst-1-ene-17-one <sup>4</sup>.

## **WADA Technical Letter – TL18**

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Laboratories should consider the following course of actions before reporting an *Adverse Analytical Finding (AAF)* for testolactone:

1. Perform a Confirmation Procedure 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* [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*: at least one of these two 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.

Should you have any further questions, please do not hesitate to contact the WADA Science Department.

### **REFERENCES**

1. Kozłowska, E. *et al.* Biotransformation 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. <https://www.wada-ama.org/en/what-we-do/science-medical/laboratories>
3. Charney, W. *et al.* Microbial transformations of steroids: a handbook. Academic Press, 2014.
4. Van Eenoo, P., & 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.