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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 EXOGENOUS COMPOUNDS IN URINE *SAMPLES*

1.0 Introduction

WADA wishes to draw the attention of the Laboratories to the following issues that may affect Laboratory operations. This pertains, in particular, to the various cases of formation of prohibited steroids or *Metabolites* originating from steroids normally present in urine *Samples* (*i.e.* non-prohibited sources) as a result of enzymatic (microbial) activities.

The impact of these metabolic *in situ* biotransformations may be different between the "A" and the "B" *Samples*. Depending on microbes' nature and growth, less frequently observed reactions could happen (*e.g.* formation of testosterone, Δ^1-dehydrogenated steroids). Consequently, the formation of the 5α- and 5β-androstanediones (free form), the most common indicator of microbial modifications of the urinary steroids, is not always observed.

The greater sensitivity of the GC-MS/MS instruments permits the detection and confirmation of steroids that may have been formed by *in situ* enzymatic reactions. Therefore, Laboratories should be cautious when detecting low levels of steroids that could be formed microbially, particularly in the absence of their major *Metabolites*. In fact, screening for steroids (parent compounds) that are expected to be extensively metabolized following administration (*e.g.* nandrolone, androstenedione, boldione) should be carefully considered as this may cause incorrect interpretations and consequently erroneous decisions. For such *Prohibited Substances*, appropriate *Metabolites* (*e.g.* 19-NA for 19-norsteroids [1]) should be targeted for analysis. It should also be borne in mind that performing an enzymatic hydrolysis (especially overnight) directly on a urine *Sample* without a preliminary extraction, may exacerbate already present microbial activity and increases the risks of side-reactions.

The global pattern of *Metabolites* must always be evaluated by the Laboratory. For example, the presence of boldione without boldenone and its other main *Metabolites* should alert the Laboratory and trigger more investigations (*e.g.* verifying that the steroids are in the conjugated form and not free, performing GC/C/IRMS, when possible, in accordance with the TD IRMS [2]).

Examples of these biotransformations include:

i. The formation of androst-4-ene-3,17-dione, 5α- and 5β-androstanediones;

ii. The formation of Δ^1 steroids such as boldenone, boldione and their *Metabolites*, androst-1-ene-3,17-diol from endogenous steroids [3, 4], as well as the formation of prednisone and prednisolone from endogenous cortisone and cortisol [5], respectively;

iii. The formation of 19-norsteroids from demethylation of endogenous steroids (*e.g.* 19-NE from etiocholanolone, 19-NA from androsterone [6]);

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iv. The formation of testosterone (free form, reported in some *Samples*; most frequently from female *Athletes*);

v. The formation of reduced (5α- and 5β-) *Metabolites* from 17α-methyltestosterone when added as an internal standard (see TL08 [7]).

Excepting iii., where it has been shown that the conversion to 19-norsteroids occurs with glucuroconjugated steroids, the steroids produced will usually be found in the free form.

2.0 Analysis and Reporting Requirements

Laboratories should consider the following course of actions in the presence of steroid *Metabolites* that may have been formed by microbial activity:

- Check for signs of microbial activity [*e.g.* ratio of 5α-androstanedione (5αAND) to Androsterone (A), ratio of 5β-androstanedione (5βAND) to Etiocholanolone (Etio)] during the Initial *Testing* Procedure (ITP);

- During the Confirmation Procedure (CP), verify the presence of *Metabolites* in the free form instead of their expected conjugated state (*e.g.* testosterone in free form);

- Perform a CP using extraction prior to enzymatic hydrolysis to avoid inducing enzymatic conversions from microbes already present in the *Samples*. Do not add internal standards that may convert into the *Prohibited Substances*;

  [Comment: In principle, it is recommended that Laboratories incorporate solid phase extraction (SPE) to clean up the Sample prior to the enzymatic hydrolysis in their chromatographic-mass spectrometric CPs. However, if the side products have already been formed prior to the enzymatic hydrolysis, ***SPE will have no impact****.]*

- Verify the presence of expected *Metabolites* according to known substance metabolic profiles.

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3.0 References

[1] *WADA Technical Document* TD NA: Harmonization of Analysis and Reporting of 19-Norsteroids related to Nandrolone.

[2] *WADA Technical Document* TD IRMS: Detection of Synthetic Forms of *Prohibited Substances* by GC/C/IRMS.

[3] Schänzer W., *et al*. Endogenous production and excretion of boldenone (17β-hydroxyandrosta- 1,4-dien-3-one), an androgenic anabolic steroid, in: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), *Recent Advances in Doping Analysis 2* Sport und Buch Strauß, Cologne, 1994; 211.

[4] Verheyden K., *et al*. Excretion of endogenous boldione in human urine: influence of phytosterol consumption. *J Steroid Biochem Mol Biol*. **117**(1-3): 8-14, 2009.

[5] Fidani M. *et al*. Presence of endogenous prednisolone in human urine. *Steroids* **78**(2): 121-126, 2013.

[6] Grosse J *et al*. Formation of 19-norsteroids by *in situ* demethylations of endogenous steroids in stored urine samples. *Steroids* **70**: 499-506, 2005.

[7] *WADA* Technical Letter TL08: Use of Internal Standards.

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