WORLD ANTI-DOPING AGENCY
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WADA Technical Letter – TL10

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Written by:	*WADA* LabEG	Approved by:	*WADA* LabEG*
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**The approval by the WADA Executive Committee is applicable only to Technical Letters issued after November 2019.*

IN SITU FORMATION OF EXOGENOUS COMPOUNDS IN URINE *SAMPLES*

The *World Anti-Doping Agency* 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 substances, appropriate *Metabolites* (*e.g.* 19-NA for 19-norsteroids) 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).

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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 [1,2], as well as the formation of prednisone and prednisolone from endogenous cortisone and cortisol [3][3], respectively;

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

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.

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.

While most of these cases would be clarified by the mandatory requirement to perform GC/C/IRMS analysis (in accordance with the TDNA, TDEAAS and TDIRMS, as applicable), the possible *in situ* formation of prednisone and prednisolone has not been addressed yet in the Technical Documents currently in effect.

Therefore, it is recommended that prior to reporting a result as an *Adverse Analytical Finding* for prednisone and/or prednisolone at levels higher than the reporting limit of 30 ng/mL and lower than 60 ng/mL, a note should be included stating that the results could be due to enzymatic activity (microbial degradation). Laboratories should take appropriate measures to evaluate whether microbial activity reasonably led to the formation of prednisone and prednisolone (in particular, in the absence of their 20β-hydroxy metabolite, absence of cortisol and cortisone, which should be present in the *Samples*). As for other steroids, GC/C/IRMS analysis may be needed to confirm the exogenous origin of prednisone and prednisolone in urine.

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[1] 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, p. 211.

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

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

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

1. 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;
2. During the Confirmation Procedure, verify the presence of *Metabolites* in the free form instead of their expected conjugated state (*e.g.* testosterone in free form);
3. For AAS and glucocorticoids, perform a Confirmation Procedure using extraction[4] 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 AAS or glucocorticoids;
4. Verify the presence of the expected *Metabolites* (*e.g.* the presence of 20β-hydroxyprednisolone).

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

[4] 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 Confirmation Procedures of AAS and glucocorticoids. However, if the side products have already been formed prior to the enzymatic hydrolysis, **SPE will have no impact**.