WADA Technical Document – TD2016NA

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HARMONIZATION OF ANALYSIS AND REPORTING OF 19-NORSTEROIDS RELATED TO NANDROLONE

1.0 Introduction

This document has been established to harmonize the Confirmation Procedure for the analysis and reporting of findings for 19-norsteroids related to nandrolone by Laboratories.

The detection of the *Use* of nandrolone (19-nortestosterone) and other 19-norsteroids (*e.g.* 19-norandrostenedione, 19-norandrostenediol) is based primarily upon the identification of the main urinary *Metabolite*, 19-norandrosterone (19-NA) at a concentration (derived from hydrolysis with β-glucuronidase from *E. coli*) greater than the Decision Limit (DL), as documented in the Technical Document on Decision Limits for the Confirmatory Quantification of Threshold Substances (TDDL) [1]. More than one *Metabolite* of administered norsteroids may be detected in urine *Samples* and reported [*e.g.* 19-noretiocholanolone (19-NE)]; however, the identification and quantification of 19-NA, including the demonstration, when required, that the 19-NA does not come from endogenous origin, is sufficient to report an *Adverse Analytical Finding* (*AAF*).

Under specific circumstances, as described below, additional Analytical *Testing* and reporting may be required.

2.0 Confirmation Procedure

2.1 Identification and Quantification

In addition to meeting the identification criteria described in the IDCR Technical Document [2], the Laboratory shall demonstrate that the concentration of 19-NA is above the DL as set out in the TDDL [1] and/or that the 19-NA detected is not of endogenous origin (*e.g.* through GC/C/IRMS analysis).

The Confirmation Procedure to determine the concentration of 19-NA in the *Sample* shall include the following characteristics:

- A deuterated internal standard (*e.g.* 19-NA-d_4-glucuronide);
- If the 19-NA concentration in the *Sample* was estimated at or below 15 ng/mL during the Initial Testing Procedure: a calibration curve at an appropriate range bracketing the estimated concentration of the analyte in the *Sample*;
- If the 19-NA concentration in the *Sample* was estimated above 15 ng/mL during the Initial Testing Procedure: a single calibration point at 15 ng/mL;

- The use of appropriate negative and positive quality control (QC) samples.

The GC/C/IRMS method shall include the following characteristics:

- Each analysis by GC/C/IRMS shall include:
  - a negative QC urine: $\delta^{13}C$ values of 19-NA and endogenous reference compound (ERC) in a normal endogenous range (*i.e.* greater than -27‰), with a difference in $\delta^{13}C$ values ($\Delta\delta$) between ERC and 19-NA lesser than 3‰; and
  - a positive QC urine: $\delta^{13}C$ value of ERC in a normal endogenous range (*i.e.* greater than -27‰), with a $\Delta\delta$ between ERC and 19-NA greater than 3‰.

  These controls shall be subjected to the same sample preparation procedure as the *Sample* Aliquot.
- The GC/C/IRMS analysis shall include the confirmation of the 19-NA peak identity[1].

2.2 Additional Tests

2.2.1 Test for Norethisterone and Pregnancy

19-NA may be excreted in small concentrations as a minor *Metabolite* of norethisterone [3], a progestogen agent of permitted use present in some oral contraceptives, and during pregnancy. Therefore, when the measured concentration of 19-NA exceeds the DL in the urine *Sample* of a female *Athlete,* the Laboratory shall perform:

- an analysis for the use of norethisterone-based contraceptives (*e.g.* detection of tetrahydronorethisterone), and if negative
- an analysis for pregnancy [*e.g.* based on the measurement of urinary human Chorionic Gonadotrophin (hCG)].

[1] For example, confirmation by GC/MS analysis performed under comparable chromatographic conditions. The purpose is to produce a chromatogram with similar peak profiles so that the spectra can be used to identify the peak(s) of interest. Minor differences in retention time between the two techniques are expected.

2.2.2 Test for demethylation

In addition, but rarely, 19-NA may be produced in urine *Samples*, in small concentrations, by *in-situ* 19-demethylation of androsterone (A) [4]. The reaction being more efficient with the 5β-isomer (*i.e.* 19-NE), such *Samples* show a less than usual ratio of 19-NA to 19-NE (*i.e.* 19-NA/19-NE less than 3.0), which is also less than the ratio of their respective urinary precursors A/E (Androsterone/Etiocholanolone)[2]. This possible endogenous formation of 19-NA can be verified by GC/C/IRMS analysis [5, 6].

2.2.3 GC/C/IRMS tests

GC/C/IRMS analysis shall be performed in the following cases[3]:

- *Samples* in which the 19-NA concentrations are measured between the DL and 10 ng/mL, except in cases of pregnancy or use of norethisterone;
- In cases of pregnancy, when the 19-NA concentration is measured greater than 15 ng/mL[4].

Furthermore, a Laboratory may perform GC/C/IRMS analysis on *Samples* containing 19-NA at concentrations below the DL, as stipulated in an existing agreement with the *Testing* Authority, or upon consultation with the *Testing* Authority, or if requested by the *Testing* Authority or *WADA*. In such cases, a positive GC/C/IRMS analysis showing the presence of 19-NA of exogenous origin is sufficient evidence to report an *AAF*.

Laboratories that do not have the analytical capacity to perform GC/C/IRMS analysis for 19-NA shall have *Samples*, for which GC/C/IRMS analysis is mandatory, transferred to and analyzed by another Laboratory that has such analytical capacity.

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[2] In the absence of inhibitors of 5α-reductase (*e.g.* finasteride).

[3] To reject the hypothesis of endogenous 19-NA formation the following criteria, based on the application of GC/C/IRMS analysis, shall be met simultaneously:

i- The Δδ value between the endogenous reference compound (ERC) [*e.g.* A or Pregnanediol (PD)] and 19-NA, *i.e.* $\Delta\delta = \delta_{ERC} - \delta_{19\text{-}NA}$, is greater than $3\,^{o}/_{oo}$, and

ii- The standard combined uncertainty (u_c) associated with the determination of $\delta^{13}C$ values, as estimated by the Laboratory during the GC/C/IRMS method validation, is not greater than $1.0\,^{o}/_{oo}$ (u_{c_Max}).

[4] In cases of pregnancy, when the concentration of 19-NA measured in a urine *Sample* is between the DL and 15 ng/mL, the IRMS analysis may also be performed to ascertain the endogenous origin of 19-NA.

Due to the occurrence of preparations of norsteroids with a carbon isotopic signature ($^{13}C/^{12}C$) close to that of endogenous human urinary steroids, the result of the GC-C-IRMS analysis of the produced 19-NA may not readily indicate its exogenous origin (*e.g.* $\delta_{19\text{-NA}}$ = -24 ‰). Therefore, in *Samples* from non-pregnant females or males, when the concentration of 19-NA is greater than the DL and the result of the GC/C/IRMS analysis is negative (*i.e.* not consistent with an exogenous origin of 19-NA) or inconclusive, the Laboratory shall determine the 19-NA/19-NE ratio based on the relative signals from the GC/MS analysis. This ratio may serve as a possible indicator of the administration of 19-norsteroids [6].

2.3 "B" *Sample* Confirmation Procedure

- In all cases, when the *AAF* for the "A" *Sample* is based on the results of a GC/C/IRMS analysis, the "B" *Sample* Confirmation Procedure also requires the GC/C/IRMS analysis (and confirmation of the 19-NA peak identity but not its quantification);
- In all other cases, the "B" *Sample* Confirmation Procedure requires the identification and quantification of the 19-NA reported. However, when the concentration of 19-NA exceeds 15 ng/mL, comparison to a single standard at 15 ng/mL and confirmation of the 19-NA peak identity are sufficient.

3.0 Interpretation

3.1 Adjusted Threshold

Only in the case of urine *Sample*s measured with a specific gravity (SG) greater than 1.020 (as determined by the Laboratory), an adjustment to the Threshold (T) shall be made to take into account the SG of the *Sample* using the following formula:

$$T_{adj} = \frac{(SG_{Sample} - 1)}{(1.020 - 1)} \cdot T$$

3.2 Decision Limit for 19-NA

The DL for 19-NA applicable to *Samples* with a SG of 1.020 or smaller is published in the TDDL [1]. In cases where the SG is greater than 1.020, the DL shall be determined for the individual 19-NA test result using the SG-adjusted T and the correspondingly adjusted guard band *g* (*i.e.* the u_{c_Max} shall be applied to the SG-adjusted T) in accordance with the TDDL [1]. Consequently, the adjusted DL shall be included in the Laboratory Test Report.

4.0 Reporting

The Laboratory shall report 19-NA detected in a *Sample* from a male or a female *Athlete* (see sections 3.1 and 3.2 above) as defined below:

A. *Samples* from pregnant female *Athletes*

No reference to the pregnancy status of an *Athlete* shall be reported in any case.

- *Adverse Analytical Finding (AAF):*
  - *Samples* for which the GC/C/IRMS analysis (see section 2.2.3 and footnote 4 above) is consistent with the exogenous origin of 19-NA.

  [The results of the 19-NA determination[5] and the GC/C/IRMS analysis[6] shall be included in the Test Report].

- *Atypical Finding* (*ATF*):
  - *Samples* for which the 19-NA concentration is greater than 15 ng/mL and the mandatory GC/C/IRMS analysis (see section 2.2.3 above) is inconclusive or not consistent with an exogenous origin of 19-NA.

  [The results of the 19-NA determination[5] and the GC/C/IRMS analysis[6] shall be included in the Test Report].

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[5] Reported 19-NA concentrations shall be expressed as follows:

- When the 19-NA concentration is greater than 15 ng/mL, no quantification is required in the Confirmation Procedure. The application of a single calibration point at 15 ng/mL is sufficient to confirm the estimated 19-NA concentration. The result shall be expressed as ">15 ng/mL" without the need for reporting the estimated concentration.
- When the 19-NA concentration is determined to be between the DL and 15 ng/mL, quantification using a calibration curve is required in the Confirmation Procedure. The confirmed concentration shall be expressed as the mean of triplicate determinations. The reported mean concentration shall be rounded **down** to one decimal place (*e.g.* a result of 2.67 ng/mL shall be reported as "2.6 ng/mL").

In every case, in accordance with the TDDL [1], the Laboratory shall report the DL for 19-NA and the combined standard uncertainty (u_c) estimated by the Laboratory at the Threshold limit.

Where the SG of the *Sample* is greater than 1.020, the value of the adjusted DL and the SG shall be included in the Laboratory Test Report *e.g.* "The concentration of 19-NA was found to be *x.x* ng/mL which is greater than the DL of *y.y* ng/mL which has been adjusted for the measured SG of 1.0zz".

[6] The Test Report for the GC/C/IRMS analysis shall include a comment indicating whether or not the GC/C/IRMS finding is consistent with an exogenous origin of 19-NA, the $\delta^{13}C$ values for 19-NA and ERC as well as the associated u_c, expressed in units.

- "No *Prohibited Substance* or *Method* on the test menu was detected":
  - No other *Prohibited Substance* or *Prohibited Method* has been confirmed in the *Sample*, and
  - *Samples* for which the 19-NA concentration is equal to or less than 15 ng/mL and the GC/C/IRMS analysis is either not performed or inconclusive/not consistent (see footnote 4 under section 2.2.3 above) with the exogenous origin of 19-NA.

B. *Samples* from female *Athletes* using norethisterone

- *Atypical Finding* (*ATF*):
  - *Samples* for which the 19-NA concentration is greater than 10 ng/mL.

    [The results of the 19-NA determination[5] shall be included in the Test Report. In addition, a comment shall be added describing the finding that demonstrates the use of norethisterone (*e.g.* "19-norandrosterone (19-NA) was found in the *Sample* at a concentration 'X'. Tetrahydronorethisterone, a *Metabolite* of norethisterone, was also found in the *Sample*)].

- "No *Prohibited Substance* or *Method* on the test menu was detected":
  - No other *Prohibited Substance* or *Prohibited Method* has been confirmed in the *Sample*, and
  - *Samples* for which the 19-NA concentrations is equal to or less than 10 ng/mL.

    [**In this case, no reference to the use of norethisterone shall be included in the Test Report**]

C. *Samples* from male or female *Athletes* (neither pregnant nor using norethisterone)

- *Adverse Analytical Finding (AAF):*
  - *Samples* for which the 19-NA concentration is greater than 10 ng/mL.

    [The results of the 19-NA determination[5] shall be included in the Test Report. In addition, for female *Athletes*, a comment shall be added explaining that pregnancy and norethisterone tests were performed and the result is not consistent with any of those conditions (*e.g. "the 19-NA finding is not consistent with pregnancy or the use of norethisterone"*);

- *Samples* for which the 19-NA concentration is equal to or less than 10 ng/mL and the GC/C/IRMS analysis (see section 2.2.3 above) is consistent with an exogenous origin of 19-NA.

  [The results of the 19-NA determination[5] and the GC-C-IRMS analysis[6] shall be included in the Test Report. In addition, for female *Athletes*, a comment shall be added explaining that norethisterone tests were performed and the result is not consistent with its use (*e.g. "the 19-NA finding is not consistent with the use of norethisterone"*)].

- *Atypical Finding* (*ATF*):
  - *Samples* for which the 19-NA concentration is between the DL and 10 ng/mL and the GC/C/IRMS analysis (see section 2.2.3 above) is inconclusive or not consistent with an exogenous origin of 19-NA, and the 19-NA/19-NE ratio is greater than 3.0.

    [The results of the 19-NA determination[5], the GC/C/IRMS analysis[6] and the 19-NA/19-NE ratio determination shall be included in the Test Report. A comment shall be added explaining that the GC/C/IRMS analysis was inconclusive (*e.g.* due to the presence of interfering compound(s) or any other factor preventing a reliable GC/C/IRMS measurement) or not consistent with an exogenous origin of 19-NA. In addition, for female *Athletes*, a comment shall be added explaining that pregnancy and norethisterone tests were performed and the result is not consistent with any of those conditions (*e.g. "the 19-NA finding is not consistent with pregnancy or the use of norethisterone"*)].

- "No *Prohibited Substance* or *Method* on the test menu was detected":
  - No other *Prohibited Substance* or *Prohibited Method* has been confirmed in the *Sample*, and
  - *Samples* for which the 19-NA concentration is equal to or less than 10 ng/mL and the GC/C/IRMS analysis (see section 2.2.3 above) is either not performed (if the concentration of 19-NA is less than the DL) or inconclusive/not consistent with an exogenous origin of 19-NA, and the 19-NA/19-NE ratio is less than 3.0.

5.0 References

1. WADA Technical Document TDDL: Decision Limits for the Confirmatory Quantification of Threshold Substances.

   https://www.wada-ama.org/en/resources/search?f[0]=field_resource_collections%3A30

2. WADA Technical Document TDIDCR: Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes.

   https://www.wada-ama.org/en/resources/search?f[0]=field_resource_collections%3A30

3. Walker CJ, Cowan DA, James VHT, Lau JCY, Kicman AT. Doping in sport—1. Excretion of 19-norandrosterone by healthy women, including those using contraceptives containing norethisterone. *Steroids* 2009; 74: 329-35.

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6. Guay C, Goudreault D, Schänzer W, Flenker U, Ayotte C. Excretion of norsteroids' phase II metabolites of different origin in human. *Steroids* 2009; 74: 350-8.

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Annex A – Flowchart for 19-NA findings