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Detection of synthetic forms of Endogenous Anabolic Androgenic Steroids by GC-C-IRMS

1.0 Introduction

This Technical Document describes the analytical method to detect the presence of synthetic forms of endogenous anabolic androgenic steroids (EAAS) by Gas Chromatography – Combustion - Isotope Ratio Mass Spectrometry (GC-C-IRMS) in urine *Samples*.

Consideration is also given to boldenone and to formestane[1], which may be naturally found in urine *Samples* at low concentrations. 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) are considered in a separate Technical Document [1] and the technical recommendations and requirements described herein shall not be applied to their analysis.

1.1 Application of GC-C-IRMS

GC-C-IRMS analyses shall be conducted as a Confirmation Procedure when the Laboratory receives an "Atypical Passport Finding (ATPF) Confirmation Procedure Request" or a "Suspicious Steroid Profile Confirmation Procedure Request"[2] notification through *ADAMS*, as described in the Technical Document on the Measurement and Reporting of EAAS (TDEAAS) [2].

In addition, a GC-C-IRMS analysis can be requested to be performed on any urine *Sample* by the Testing Authority, the *Athlete* Passport Management

[1] Formestane (4-hydroxyandrost-4-en-3,17-dione) is an aromatase inhibitor but its structure is similar to EAAS and it also may be found naturally in urine *Samples*; therefore, it requires a similar Analytical Testing approach as EAAS.

[2] The Laboratory shall receive the automatic "Suspicious Steroid Profile Confirmation Procedure Request" notification through *ADAMS* 14 calendar days after *Sample* reception. The Laboratory shall proceed with the GC-C-IRMS Confirmation Procedure unless, after contacting the Testing Authority, the Testing Authority can justify that the GC-C-IRMS analysis is not necessary. If no feedback is received from the Testing Authority within 7 calendar days, the Laboratory shall proceed with the GC-C-IRMS confirmation analysis.

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Unit (APMU), or *WADA*, even if the *Markers* of the "steroid profile" are within the normal ranges.

Furthermore, the Laboratory may at any time advise[3] the Testing Authority to perform (or not) GC-C-IRMS analyses based upon its expertise, for example in the presence of any other *Marker* of administration of EAAS such as 6α-hydroxyandrostenedione, 3α,5-cyclo-5α-androstan-6β-ol-17-one, 6β-hydroxyandrosterone or 6β-hydroxyepiandrosterone (sulfates), or an altered ratio of 7β-hydroxydehydroepiandrosterone to 16α-hydroxyandrosterone (sulfates).

1.1.1 GC-C-IRMS analysis for formestane, boldenone or boldenone metabolite(s)

In *Samples* containing formestane, boldenone or boldenone metabolite(s), the GC-C-IRMS analysis for these compounds shall be conducted before reporting an *Adverse Analytical Finding* when their estimated SG-adjusted[4] concentrations are as follows:

- Concentration of formestane between 50 ng/mL and 150 ng/mL.
- Concentration of boldenone and/or its metabolite(s) between 5 ng/mL and 30 ng/mL.

Laboratories that do not have the analytical capacity to perform GC/C/IRMS analysis for formestane and/or boldenone or boldenone metabolite(s) shall have the *Sample* analyzed by another Laboratory that has such analytical capability.

Findings for boldenone and/or its metabolite(s) at concentrations estimated below 5 ng/mL (SG-adjusted[4], if needed) are to be considered as *Atypical Findings*, unless the results of the GC-C-IRMS analysis, if performed (depending on Laboratory's analytical capacity and following consultation with the Testing Authority), conclusively establish the exogenous origin of the substance (*Adverse Analytical Finding*).

[3] Or as covered by contractual agreement between the Laboratory and the Testing Authority.

[4] When the SG of the urine *Sample* is greater than 1.020, the concentrations are adjusted to a SG of 1.020 based on the following equation (free and hydrolyzed glucuroconjugated steroids).

$$\mathbf{Conc_{corr} = Conc_{measured} * (1.020 - 1)/(SG - 1)}$$

Concentrations for formestane below 50 ng/mL (SG-adjusted[4], if needed) are to be considered as negative, unless the results of the GC-C-IRMS analysis, if performed (depending on Laboratory's analytical capacity and following consultation with the Testing Authority), conclusively establish the exogenous origin of the substance (*Adverse Analytical Finding*).

Findings above 30 ng/mL for boldenone and/or its metabolite(s) or above 150 ng/mL for formestane (SG-adjusted[4]) shall be considered as *Adverse Analytical Findings* without the need for GC-C-IRMS analysis.

1.1.2 B Sample Confirmation Procedure

When an *Adverse Analytical Finding* is reported for the *Markers* of the "steroid profile" or for non-threshold substances such as formestane, boldenone or boldenone metabolite(s) based on the results of a GC-C-IRMS analysis performed on the A *Sample*, the GC-C-IRMS analysis shall also be performed during the B *Sample* Confirmation Procedure, if applicable.

2.0 GC-C-IRMS analysis

The application of GC-C-IRMS is based on the determination of the $\delta^{13}C$ value of urinary metabolites or target compounds (TC) (*e.g.* Androsterone (A), Etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol), Testosterone (T), Epitestosterone (E), boldenone, formestane and others) and the difference in $\delta^{13}C$ values, *i.e.* the $\Delta\delta^{13}C$ value, between the endogenous reference compound(s) (ERC) and the TC.

The GC-C-IRMS analysis shall be conducted in a single *Sample* Aliquot.

2.1. GC-C-IRMS Method Characteristics

Laboratories shall implement the following in their GC-C-IRMS methodology:

- As part of the method validation, the Laboratory shall determine the range of peak intensities for each analyte that gives a consistent $\delta^{13}C$ value (signal independency, linearity).
- The system shall be calibrated periodically against a steroid Reference Material (RM) (*e.g.* CU/USADA 34, CU/USADA 35, or other mixture of steroid(s)) that is traceable to the assigned values of the recognized international RM. Major revisions of the system (*e.g.* change of

reference gas, cleaning of the ion source, etc.) shall require calibration of the system.

- The stability of CO_2 pulses shall be tested before the analysis of each batch of *Samples*. The linearity of the signal (with pulses of CO_2) shall be checked regularly, *e.g.* monthly.
- The urinary TC(s) and ERC(s) once hydrolyzed shall be further purified by High Performance Liquid Chromatography (HPLC) (recommended), Solid Phase Extraction (SPE) or other equivalent purification step prior to the GC-C-IRMS analysis.
- The following controls and RMs shall be included in each batch of *Samples* analyzed and subjected to the same *Sample* preparation procedure:
  - A negative[5] and a positive urinary control sample. The relevant TC shall meet the positivity criteria in the positive urine control sample.
  - An appropriate RM for the relevant TC(s) and ERC(s), with known $\delta^{13}C$ value(s).
- Laboratories shall be capable of performing GC-C-IRMS analyses on A, Etio, 5αAdiol, 5βAdiol, T and E. When the concentration is sufficient, the TC(s) should be selected/prioritized depending on the variable(s) of the "steroid profile" that prompted the GC-C-IRMS analysis.
- T, 5αAdiol and/or 5βAdiol are the preferred TC(s) to detect the administration of T.
- The analysis should be based on the use of pregnanediol (PD) as the principal ERC. However, another ERC, either 5α-androst-16-en-3α-ol (16-en), 11β-hydroxyandrosterone (11-OHA) or 11-keto-etiocholanolone (11-O-Etio) should also be routinely used, since PD may be suppressed, affected by poor chromatography or by the administration of pregnenolone. The same ERC shall be used for the determination of all the $\Delta\delta^{13}C$ values.
- No value obtained from peaks of intensity below or above the range of linearity or in the presence of significant co-eluting peaks shall be considered or reported.

[5] This does not apply to GC-C-IRMS determinations for boldenone and formestane.

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- As estimated from validation experiments, the combined standard measurement uncertainty (u_c) for the determination of the $\delta^{13}C$ values shall be not higher than 1.0 $^0/_{00}$ (u_{c_Max}).
- The Laboratory shall determine the $\Delta\delta^{13}C$ values for each ERC-TC pair analyzed in a population of volunteers and *Athlete* negative *Samples* (a minimum of 20 male and 20 female urine samples)[5].
- The steroids may be analyzed underivatized or after acetylation, but only values equivalent to free compounds shall be used to determine the $\Delta\delta^{13}C$ value of the ERC-TC pair. The following mass balance equation for adjustment of the measured $\delta^{13}C$ values from acetates back to the free form shall be used:

$$\delta C_s = (n_{cd}\delta C_{cd} - n_d\delta C_{dcorr}) / n_s$$

where n: number of carbon atoms; s: native steroid (underivatised form); d: derivative group (*e.g.* acetyl), and cd: derivatised compound.

As δC_d is not known, δC_{dcorr} is estimated empirically by consecutive measurements of a non-acetylated and acetylated steroid (*e.g.* 5αAdiol, 5βAdiol or PD).

2.2 Identification of urinary metabolites prior to reporting an Adverse Analytical Finding

- A GC-MS analysis is required to ensure the identity of the peaks of the relevant TC(s) and ERC and the absence of significant interference prior to reporting an *Adverse Analytical Finding* based on GC-C-IRMS results. This is not necessary when the GC-C-IRMS results are inconclusive or negative.
- The same mixtures shall be analyzed by GC-MS under similar chromatographic conditions. Minor differences in retention times (RT) between the two techniques are expected. The provisions of the Technical Document on Identification Criteria (TDIDCR) shall be followed [3].
- In cases when the GC-C-IRMS analysis demonstrates the exogenous origin of TC(s), the Laboratory shall confirm the relevant *Marker(s)* of the "steroid profile" following the Confirmation Procedure described in the TDEAAS [2].

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2.3 Interpretation of GC-C-IRMS results

The results of the GC-C-IRMS analyses shall be interpreted as follows:

Positive

When $\Delta\delta^{13}C$ value(s) are consistent with the exogenous origin of the TC(s), *i.e.* if one of the following sets of criteria is met[6] (**Appendix 1**):

i. The $\Delta\delta^{13}C$ value of the pair ERC-T, and one of the pairs ERC-5αAdiol or ERC-5βAdiol shall be greater than 3 $^{0}/_{00}$ in males or in females provided that the concentration of the TC and ERC is within the linear range of the method.

ii. The $\Delta\delta^{13}C$ value of both pairs ERC-5αAdiol and ERC-5βAdiol shall be greater than 3 $^{0}/_{00}$.

iii. For E, when the concentration is greater than 50 ng/mL (SG-adjusted), the $\Delta\delta^{13}C$ value of the pair ERC-E shall be greater than 4 $^{0}/_{00}$.

iv. The $\Delta\delta^{13}C$ value of the pair ERC-A or ERC-Etio shall be greater than 3 $^{0}/_{00}$ and 4 $^{0}/_{00}$, respectively.

v. Alternatively, if the $\Delta\delta^{13}C$ value of the pair ERC-A is between 2 $^{0}/_{00}$ and 3 $^{0}/_{00}$ and/or the $\Delta\delta^{13}C$ value of the pair ERC-Etio is between 3 $^{0}/_{00}$ and 4 $^{0}/_{00}$, the $\Delta\delta^{13}C$ value of one of the pairs ERC-5αAdiol or ERC-5βAdiol shall be greater than 3 $^{0}/_{00}$.

vi. The $\Delta\delta^{13}C$ value of the ERC-5αAdiol pair shall be greater than 4 $^{0}/_{00}$ in combination with the $\delta^{13}C$ value of the 5αAdiol being equal or lower than -27 $^{0}/_{00}$ (*e.g.* DHT administration).

vii. The $\Delta\delta^{13}C$ value of the pair ERC-formestane, ERC-boldenone and/or ERC-boldenone metabolite shall be greater than 4 $^{0}/_{00}$.

For all of the above cases i) to vi), the $\Delta\delta^{13}C$ value of the diagnostic ERC-TC pair in the *Sample* shall be greater than the mean $\Delta\delta^{13}C$ + 3 standard deviations (SD) value of that pair in the population of negative samples measured by the Laboratory.

[6] It is not expected that all metabolites will be affected to the same extent. Decisions based on the $\Delta\delta^{13}C$ criteria specified in i) to vii) take into account the measurement uncertainty associated with the contributing $\delta^{13}C$ values.

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Negative

When $\Delta\delta^{13}C$ values do not confirm the exogenous origin of the TCs, *i.e.* when the $\Delta\delta^{13}C$ values of the ERC-TC pairs are within the mean $\Delta\delta^{13}C$ + 3 SD value for the population of negative samples measured by the Laboratory.

Inconclusive

i) When only one of the combined criteria specified in points i), ii), v) or vi) above is met (*e.g.* $\Delta\delta^{13}C$ value for the pair ERC-T > 3 $^{0}/_{00}$ but $\Delta\delta^{13}C$ for both pairs of ERC-Adiols < 3 $^{0}/_{00}$).

ii) Due to technical limitations *e.g.* when there is insufficient *Sample* volume or very low concentrations of TCs or ERCs, or in the presence of interfering compounds or any other factor preventing a reliable measurement of the relevant diagnostic metabolite or ERC-TC pair.

iii) The Laboratory can interpret the results as inconclusive when the criteria for reporting an *Adverse Analytical Finding* are not met but in its opinion are neither consistent with the endogenous origin of the urinary metabolites (*e.g.* ERC $\delta^{13}C$ value at -24.5 $^{0}/_{00}$ and TC at -27.0 $^{0}/_{00}$).

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3.0 Reporting GC-C-IRMS Results

The Laboratory shall report the results of the GC-C-IRMS analyses as follows:

1. Adverse Analytical Finding

Samples for which the results of the GC-C-IRMS analysis were positive:

- Each *Sample* for which an *Adverse Analytical Finding* is reported shall be reported individually.
- The Test Report shall include:
  - A comment indicating that the GC-C-IRMS finding is consistent with an exogenous origin of the TC(s), specifying the identity of the TC(s) analyzed and confirmed.
  - The $\delta^{13}C$ value of the relevant TC(s) and ERC, and the associated u_c, expressed in units.
  - The confirmed values (*e.g.* concentrations, T/E ratios) of the relevant *Marker(s)* of the "steroid profile" and the associated u_c, expressed in units [2] [7].

Reporting example for the Test Report:

GC-C-IRMS results are consistent with the exogenous origin of testosterone and 5βAdiol ($\delta^{13}C$ values: T= -27.5 °/₀₀; 5βAdiol= -25.2 °/₀₀; PD= -20.2 °/₀₀; u_c = 0.8 °/₀₀). T/E = 4.5, u_c = 0.5.

Provision of a Second Opinion for GC-C-IRMS

When the results of the GC-C-IRMS analysis indicate an *Adverse Analytical Finding* for a *Sample*, the Laboratory should seek the opinion of an expert from a second Laboratory before reporting the *Adverse Analytical Finding*.

[7] When the GC-C-IRMS Confirmation Procedure is applied to formestane, boldenone or boldenone metabolite(s) only, the Laboratory does not need to perform the quantitative confirmation of these substances, or report confirmed values of the *Markers* of the "steroid profile".

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2. Atypical Finding

Samples for which the results of the GC-C-IRMS analysis were inconclusive:

- Each *Sample* for which an *Atypical Finding* is reported shall be reported individually.
- The Test Report shall include:
  - A comment indicating that the GC-C-IRMS finding is inconclusive specifying the identity of the TC(s) analyzed and confirmed.
  - The $\delta^{13}C$ value of the relevant TC(s) and ERC, and the associated u_c, expressed in units.
  - The confirmed T/E ratio and the associated u_c, expressed in units [2][7].

Reporting example for the Test Report:

The results of the GC-C-IRMS analysis for testosterone and 5βAdiol are inconclusive ($\delta^{13}C$ values: T = -27.6 °/₀₀; 5βAdiol= -26.2 °/₀₀; PD= -24.5 °/₀₀; u_c = 0.8 °/₀₀).T/E = 10.2, u_c = 0.8.

3. No Prohibited Substance(s) or Metabolite(s) or Marker(s) of a Prohibited Method(s) on the test menu were detected

Samples for which the results of the GC-C-IRMS analysis were negative:

- The Test Report shall include:
  - A comment mentioning that the GC-C-IRMS results do not indicate an exogenous origin of the TCs.
  - The confirmed T/E ratio and the associated u_c, expressed in units [2][7].

Reporting example for the Test Report:

GC-C-IRMS results do not confirm the exogenous origin of the urinary metabolites of testosterone related steroids. T/E = 7.4, u_c = 0.7.

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4.0 Interpretation

- The GC-C-IRMS and GC-MS or GC-MS/MS methods provide independent and complementary information, but their results must be considered together to bring a conclusion that is supported by the scientific literature and knowledge.
- The urinary "steroid profile" may show no major anomaly whilst being excreted following the administration of a steroid related to T; in such a case, the results of the GC-C-IRMS analysis indicating a synthetic origin of the steroid metabolites shall prevail.
- Conversely, values for variable(s) of the "steroid profile" may be outside the subject-based longitudinal reference range while being of endogenous origin (*e.g.* heavy ethanol drinking leading to an increased urinary excretion of T and 5βAdiol, microbial formation of free T, or intense, prolonged exercise increasing the excretion of A).
- The "steroid profile" may be altered by the administration of a preparation of a steroid related to T of relatively enriched $\delta^{13}C$ value, which may not be detected by GC-C-IRMS. In such cases, the provisions of the Technical Document on Results Management Requirements for the *Athlete* Biological Passport (TDRMR) shall be followed [4].

5.0 References

1. *WADA* Technical Document TD19NA: Harmonization of Analysis and Reporting of 19-Norsteroids related to Nandrolone.
http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/To_be_effective_Jan_2012/WADA_TD2012NA_Final_EN.pdf

2. *WADA* Technical Document TDEAAS: Endogenous Anabolic Androgenous Steroids: Measurement and Reporting.

http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2014-EAAS-Endogenous-Anabolic-Androgenic-Steroids-Measurement-and-Reporting-EN.pdf

3. *WADA* Technical Document TDIDCR: Identification Criteria for Qualitative Assays incorporating Column Chromatography and Mass Spectrometry.

http://www.wada-ama.org/en/Science-Medicine/Anti-Doping-Laboratories/Technical-Documents/

4. *WADA* Technical Document TDRMR: Results Management Requirements for the *Athlete* Biological Passport. Appendix E to the "Athlete Biological Passport Operating Guidelines".
http://www.wada-ama.org/Documents/Science_Medicine/Athlete_Biological_Passport/WADA-ABP-Operating-Guidelines_v4.0-EN.pdf

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Appendix 1. Interpretation criteria for GC-C-IRMS positive test

Positive Criteria Section 6.3	$\Delta\delta$ **ERC-TC** > ($\Delta\delta$ + 3SD) Population Negative Samples					$\Delta\delta$ **ERC-TC**
	T	**E**[#]	**A**	**Etio**	**5αAdiol, 5βAdiol**	**Boldenone** or **Formestane**
i.	> 3 $^{0}/_{00}$				> 3 $^{0}/_{00}$ (either Adiol)	
ii.					> 3 $^{0}/_{00}$ (both Adiols)	
iii.		> 4 $^{0}/_{00}$				
iv.			> 3 $^{0}/_{00}$			
				> 4 $^{0}/_{00}$		
v.			2-3 $^{0}/_{00}$		> 3 $^{0}/_{00}$ (either Adiol)	
				3-4 $^{0}/_{00}$	> 3 $^{0}/_{00}$ (either Adiol)	
vi.					$\Delta\delta$(ERC-5α) > 4 $^{0}/_{00}$ and $\delta(5\alpha) \leq -27$ $^{0}/_{00}$	
vii.						> 4 $^{0}/_{00}$

[#] Concentration (SG-adjusted) greater than 50 ng/mL.