Document Number:	TD2014EAAS	Version Number:	1.0
Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
Date:	11 September 2013	Effective Date:	1 January 2014

Endogenous Anabolic Androgenic Steroids
Measurement and Reporting

1.0 Introduction

The purpose of this Technical Document is to harmonize the approaches to the measurement and reporting of endogenous anabolic androgenic steroids (EAAS) in urine, including data in support of the steroidal module of the *Athlete* Biological Passport (ABP) or "steroid profile".

EAAS concentrations and their ratios form the urinary "steroid profile", which may be altered following the administration of synthetic forms of EAAS, in particular testosterone (T), its precursors [for example androstenediol, androstenedione and prasterone (dehydroepiandrosterone or DHEA)], or its active metabolite [dihydrotestosterone (DHT)], as well as epitestosterone (E).

The steroid module of the ABP uses the Adaptive Model to identify an Atypical Passport Finding (ATPF), which triggers the performance of Confirmation Procedures. It is also used to apply intelligent target *Testing* of the *Athlete* on a longitudinal basis. Furthermore, an abnormal "steroid profile" (obtained from a single urine *Sample*) or an atypical "longitudinal steroid profile" (including values obtained from a series of "steroid profiles" collected over a period of time), may be a means to pursue an anti-doping rule violation (ADRV).

EAAS *Testing* and reporting follows a two-step procedure: an Initial Testing Procedure aims to estimate the "steroid profile" in the *Athlete's Sample*. A subsequent Confirmation Procedure is performed when the estimated "steroid profile" represents an ATPF. The Confirmation Procedure includes the quantification of the *Markers* of the "steroid profile" as described in this Technical Document as well as Gas Chromatography – Combustion - Isotope Ratio Mass Spectrometry (GC-C-IRMS) analysis which is considered in a separate Technical Document.

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1.1 The "Steroid Profile"

Each urine *Sample* shall be analyzed to determine its "steroid profile".

For the purposes of this Technical Document, the "steroid profile" is composed of the following *Markers* (as free steroid content obtained from the free steroid fraction plus those released from the conjugated fraction on hydrolysis by glucuronidase):

- Testosterone (T),
- Epitestosterone (E),
- Androsterone (A),
- Etiocholanolone (Etio),
- 5α-androstane-3α,17β-diol (5αAdiol),
- 5β-androstane-3α,17β-diol (5βAdiol), and
- The ratio of Testosterone to Epitestosterone (T/E).

Other urinary steroids or ratios of steroid metabolites could be useful in evaluating a "steroid profile" (*e.g.* A/T, A/Etio, 5αAdiol/5βAdiol, 5αAdiol/E [1]).

The administration of EAAS can alter one or more of the *Markers* and/or ratios of the urinary "steroid profile", resulting in increased or decreased concentrations and/or ratios of specific pairs of steroid metabolites. Additionally, alteration of the urinary "steroid profile" can occur for a number of reasons including, but not limited to:

- A large intake of alcohol (ethanol).
- The administration of ketoconazole, human chorionic gonadotrophin (hCG) in males or of other anabolic steroids (*e.g.* stanozolol).
- The administration of inhibitors of 5α-reductase (*e.g.* finasteride).
- The use of masking agents (*e.g.* probenecid) and diuretics.
- Microbial growth.

[1] In *ADAMS*, the values of these four ratios are computed after the reporting of the "steroid profile" by the Laboratory.

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2.0 Initial Testing Procedure

In the Initial Testing Procedure, the Laboratory shall use a method validated in urine that is appropriate for estimating the *Markers* of the "steroid profile" in the range of values determined in males and females.

The Initial Testing Procedure is conducted on a single Aliquot.

2.1 Method Characteristics

- Gas chromatography combined with mass spectrometry (GC-MS or GC-MS/MS) of TMS derivatives (keto and hydroxyl groups) is required.
- Calibration standards should be analyzed periodically, and whenever a significant change is made to the analytical setup.
- A urine quality control (QC) sample containing representative levels of the analytes should be included in each sequence of analysis.
- The enzymatic hydrolysis shall be carried out with purified β-glucuronidase from *E. coli* (*H. pomatia* mixtures are not acceptable).
- The completeness of hydrolysis of the glucuroconjugated urinary steroids shall be verified with isotopically labeled A-glucuronide (or an equivalent scientifically recognized alternative).
- The completeness of the derivatization shall be verified through the monitoring of mono-O-TMS vs. di-O-TMS derivative of A.
- When needed, the volume[2] of the *Sample* Aliquot may be adjusted as a function of its specific gravity (SG) and of the gender of the *Athlete*.
- The T/E ratios shall be determined from the ratios of the corrected chromatographic peak areas or peak heights[3].
- The linearity of the method, established during method validation, shall cover the ranges of values normally found in males and females -

[2] Much lower levels of T and E are generally present in female *Samples* and in those *Samples* with low SG; therefore, larger Aliquot volumes may be required for a reliable measurement.

[3] Ratios of T and E peak heights or peak areas corrected against a calibrator or a calibration curve (same mass or same ion transition screened for both steroids).

the limit of quantification (LOQ) for T and E shall not be higher than 2 ng/mL[4].

- The relative standard combined uncertainty [u_c(%)] for the determination of A, Etio, 5αAdiol, 5βAdiol, T and E, as estimated during method validation of the Initial Testing Procedure, shall be not higher than 30% at the respective LOQ;

  For concentration values at five times the LOQ, the u_c(%) shall be not higher than 20% for A and Etio or 25% for the Adiols;

  The u_c(%) for determinations of T and E shall not exceed 20% when the steroid concentrations are higher than 5 ng/mL;

  The u_c(%) for determinations of T/E ratios calculated from the corrected chromatographic peak areas or heights shall not exceed 15% when the concentrations of T and E are higher than 5 ng/mL; for lower concentrations of T and E, the u_c(%) for the T/E determinations shall not exceed 30%.

- Evidence of microbial degradation (*e.g.* presence of 5α- and 5β-androstanedione or 4-androstenedione) and the presence of 5α-reductase inhibitors (*e.g.* finasteride) shall be monitored.

[4] The LOQ shall be determined as the lowest concentration that can be measured with the uncertainty criteria established for the given *Marker* of the "steroid profile" when applying the Initial Testing Procedure.
The LOQ for T, E, A, Etio, 5αAdiol and 5βAdiol shall be reported once in *ADAMS* by the Laboratory. The LOQ values shall be updated in *ADAMS* whenever a significant change is made to the analytical method.

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2.2. Reporting the 'steroid profile' from the Initial Testing Procedure

The Laboratory shall report in *ADAMS* the T/E ratio, the concentrations of T, E, A, Etio, 5αAdiol and 5βAdiol, the SG and the validity of the *Sample*, as determined in the Initial Testing Procedure.

The "steroid profile" shall be reported in *ADAMS* as follows:

- The concentrations of T, E, A, Etio, 5αAdiol and 5βAdiol shall be reported without adjustment for the SG of the *Sample* and to 2 significant figures (e.g. T = 5.2; 52; 520) [5].
- The T/E shall be reported to 2 significant figures (e.g. T/E = 0.12; 1.2; 12).

The validity of the *Sample* shall be reported in ADAMS as "yes" or "no".

- A *Sample* showing signs of microbial degradation or containing any of the substances[6] that may cause an alteration of the "steroid profile", as described in Section 1.0 above, may not be suitable for inclusion in the "longitudinal steroid profile". In such cases the validity of the "steroid profile" shall be reported in *ADAMS* as "no" and an explanation shall be included in the Test Report in *ADAMS*.
- When the measurement of a *Marker* of the "steroid profile" is not possible due to, for example, dilution, unusual matrix interferences, inhibition of the enzymatic hydrolysis or incomplete derivatization, the Laboratory should repeat the analysis with a modified, validated *Sample* preparation and analysis (*e.g.* solid phase extraction, extraction with a different solvent or other equivalent procedure). However, when the problem cannot be resolved, the negatively impacted variable(s) of the "steroid profile" shall be reported as "-1", the validity as "no", and a comment shall be included in the Test Report in *ADAMS* stating that the *Marker(s)* could not be measured reliably.

The Laboratory may recommend in the Test Report in *ADAMS* that a *Sample* be submitted to confirmation analyses by GC-C-IRMS.

[5] Any concentration measured below the LOQ shall be reported as -1 by the Laboratory.

[6] It is not mandatory that the Laboratory tests for the presence of ethanol metabolite(s) or ketoconazole during the Initial Testing Procedure.

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3.0 Confirmation Procedure

Confirmation Procedures for the exogenous administration of EAAS include the GC-MS or GC-MS/MS quantification and GC-C-IRMS analyses of the relevant *Marker(s)* of the "steroid profile". GC-C-IRMS analyses are considered in a separate Technical Document.

- The Laboratory shall confirm the relevant "steroid profile" *Marker(s)* or ratio (*e.g.* the T/E ratio) measured in the Initial Testing Procedure when, upon reporting the results in *ADAMS* and following the application of the Adaptive Model of the ABP to the "longitudinal steroid profile" of the *Athlete*, the Laboratory is informed through *ADAMS* of an ATPF.
- In the case when the "longitudinal steroid profile" of the *Sample* cannot be processed by the Adaptive Model in *ADAMS*, the Laboratory shall proceed with the Confirmation Procedure(s) when one of the following criteria is met[7]:
  - T/E ratio (calculated from the corrected chromatographic peak areas or heights) greater than 4.0.
  - Concentration of T or E (adjusted for the SG[8]) greater than 200 ng/mL in males or greater than 50 ng/mL in females.
  - Concentration of A or Etio (adjusted for the SG[8]) greater than 10,000 ng/mL combined with ratio of A/Etio lower than 0.4 in males (in the absence of inhibitors of 5α-reductase) or greater than 4 in either sex.

[7] If the "steroid profile" of the *Sample* cannot be processed by the Adaptive Model in *ADAMS*, the Laboratory shall receive an automatic notification from *ADAMS* 14 calendar days after *Sample* reception. The Laboratory shall proceed with the Confirmation Procedure(s) unless, after contacting the Testing Authority, the Testing Authority can justify that the Confirmation Procedure(s) is not necessary.

[8] The concentrations are adjusted to a urine SG of 1.020 based on the following equation (free and hydrolyzed glucuroconjugated steroids).

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

3.1 GC-MS or GC-MS/MS quantification Confirmation Procedure

The Laboratory shall identify (in compliance with the TDIDCR [1])[9] and quantify the relevant *Markers* of an ATPF in one additional *Sample* Aliquot by a validated fit-for-purpose GC-MS or GC-MS/MS quantification method.

- If GC-C-IRMS analysis has been performed with negative or inconclusive results, the Laboratory shall confirm the T/E ratio only.
- In cases when the GC-C-IRMS analysis demonstrates the exogenous administration of EAAS, the Laboratory shall confirm the relevant variable(s) of the "steroid profile". When the exogenous administration involves T, only the T/E ratio shall be confirmed.

During the Confirmation Procedure, the presence of conjugated metabolite(s) of ethanol or ketoconazole shall be determined as well as the signs of microbial degradation including, for example, the presence of the free forms of T, 5α- and 5β-androstanedione, 4-androstenedione, or DHEA.

3.1.1 Method Characteristics for GC-MS or GC-MS/MS quantification Confirmation Procedure

The same analytical requirements presented in 2.1 apply, with the following modifications:

- Calibration standards and urine QC samples shall be included;
- The u_c(%) shall be not higher than 15% for determinations of A, Etio, 5αAdiol and 5βAdiol at concentrations representing five times the respective LOQ;
- For determinations of T, E and T/E ratios, the u_c(%) shall be not higher than 15% when the concentrations of T and E are higher than 5 ng/mL.

[9] For T/E values, only T needs to be identified if the concentration level and volume of the *Sample* are sufficient.

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3.1.2 Reporting Results from the GC-MS or GC-MS/MS Confirmation Procedures

The Laboratory shall report in *ADAMS* the confirmed values of the "steroid profile" (without adjustment for the SG of the *Sample*), the associated u_c expressed in units and the SG of the *Sample*.

The presence of signs of microbial degradation, of conjugated metabolite(s) of ethanol, of inhibitors of 5α-reductase, or of any other substances that might have altered the "steroid profile" shall be reported.

6.0 References

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

1. *WADA* Technical Document TDIDCR (current version): Identification Criteria for Qualitative Assays incorporating Column Chromatography and Mass Spectrometry.