REPORTING AND EVALUATION GUIDANCE FOR TESTOSTERONE, EPITESTOSTERONE, T/E RATIO AND OTHER ENDOGENOUS STEROIDS

1. Introduction:

This guide has been prepared to ensure that Laboratories can report, in a uniform way, the presence of abnormal profiles of urinary steroids resulting from the administration of testosterone or its precursors, androstenediol, androstenedione, dehydroepiandrosterone (DHEA) or a testosterone metabolite, dihydrotestosterone or a masking agent, epitestosterone. It also provides guidance to the Testing Authority on how to conduct the evaluation of Adverse Analytical Findings reported by the Laboratories.

It is proven that administration of these steroids alters one or more of the parameters of the urinary steroid profile. Elevated levels of urinary metabolites, which are part of the “steroid profile”, e.g. testosterone, epitestosterone, dihydrotestosterone, androsterone, etiocholanolone, DHEA as well as other specific metabolites are not consistent with normal endogenous production and result from the intake of these steroids. Increased ratios of specific pairs of steroid metabolites are also indicative of the administration of these endogenous steroids.

It is emphasized that the following requirements shall be applied by all Laboratories in their routine practice.

2. Specific requirements for GC/MS measurement of T/E value, concentration of testosterone, concentration of epitestosterone:

The T/E value is given by the peak area or peak height ratio of testosterone and epitestosterone (equivalent to the glucuronide) obtained by measuring the ion at m/z 432 by GC/MS analysis in a Single Ion Monitoring mode (SIM). The T/E value is usually measurable regardless of the concentration of both steroids. Whether measured from the Screening Procedure or the Confirmation Procedure, it must be corrected using an appropriate standard (e.g. calibration curve, quality control sample(s) or authentic standard solutions of both testosterone and epitestosterone). The concentration of testosterone and epitestosterone (equivalent to the glucuronide) should be estimated but should not be used to determine the T/E value. In the case of high T/E values, the concentration of epitestosterone is frequently low and it may not always be possible to measure epitestosterone precisely. In such cases, only the concentration of testosterone (equivalent to the glucuronide) is to be determined.

The Screening Procedure which is normally conducted on a single aliquot shall be carried out including, together in the same batch, a control sample where the T/E value, concentrations of testosterone and epitestosterone are known.

Reference ranges of the various parameters of the urinary steroid profile have been described for populations of both males and females. It should be borne in mind that there is significant
variation between individuals. A normal level for one individual may in another be elevated and be consistent with doping. The Laboratory will adapt its testing procedures to the Sample tested; for example, female or male, Asian or Caucasian (when the information is provided). The concentration of urinary steroids such as testosterone and epitestosterone varies greatly between individuals and also depends upon the specific gravity of the urine Sample; only values corrected for a specific gravity value of 1.020 can be compared.

It is recommended that a urine Sample in which any one of the following criteria is met during the Screening Procedure, be routinely submitted to the IRMS analysis:

i) T/E value equal or greater than 4;

ii) concentration of testosterone or epitestosterone (equivalent to the glucuronide) greater than 200 ng/mL\(^1\);

iii) concentration of androsterone or etiocholanolone (equivalent to the glucuronide) greater than 10,000 ng/mL\(^1\);

iv) concentration of DHEA (equivalent to the glucuronide) greater than 100 ng/mL\(^1\).

It is recognised that other parameters may justify a need for IRMS study and the reason should be documented.

Any result that will be used to support an Adverse Analytical Finding shall be confirmed and quantified.

Confirmation of elevated T/E values, concentration of testosterone, epitestosterone or any other steroid metabolite under consideration is to be performed in triplicate. The confirmation of the identity of any steroid reported with abnormal properties must be made (refer to technical document TD2003IDCR). Appropriate calibration (e.g. calibration curve, deuterated standards, quality control samples) is to be included in the protocol of the Confirmation Procedure.

Confirmed elevated concentration of steroids will be reported as such together with the value adjusted for the specific gravity of the urine Sample using the following formula:

\[
\text{Concentration}_{1.020} \text{ ng/mL} = \frac{(1.020 - 1)}{(\text{Specific gravity of the Sample} - 1)} \times \text{Concentration measured ng/mL}
\]

The urine Sample is not collected under sterile conditions, and where the circumstances are favourable, the microbes present in the Sample can cause changes to the profile of the urinary steroids. Initially there is cleavage of the glucuronides and sulfates followed by modifications of the steroids' structure by oxido-reductive reactions. To report an Adverse Analytical Finding of an elevated T/E value, testosterone or epitestosterone concentration or any other endogenous steroid parameters, the concentration of free testosterone and/or epitestosterone in the specimen is not to exceed 5% of the respective glucuronoconjugates. Elevated amounts of 5α- and 5β-androstan-3,17-dione in the free form also indicate microbial degradation.

\(^1\) Concentrations adjusted for a specific gravity value of 1.020
3. Isotope ratio mass spectrometry:

When a parameter of the steroid profile indicates a need to further study, its $^{13}\text{C}/^{12}\text{C}$ value expressed in delta units per mil ($\delta^{13}\text{C}$) or that of its metabolites will be measured and compared to that of urinary reference steroids within the sample not affected by administration. Depending upon the nature of the endogenous steroid suspected to have been administered, the metabolites analysed could be testosterone, epitestosterone, androsterone, etiocholanolone, the androstanediols, DHEA, or other relevant metabolites while the urinary reference steroid usually analysed by the Laboratories is one of, pregnanediol, pregnanetriol, cholesterol, 11-hydroxyandrostosterone or 11-ketoetiocholanolone. The instrumentation should be calibrated with an appropriate Reference Material.

The results will be reported as consistent with the administration of a steroid when the $^{13}\text{C}/^{12}\text{C}$ value measured for the metabolite(s) differs significantly i.e. by 3 delta units or more from that of the urinary reference steroid chosen. In some Samples, the measure of the $^{13}\text{C}/^{12}\text{C}$ value of the urinary reference steroid(s) may not be possible due to their low concentration. The results of such analyses will be reported as “inconclusive” unless the ratio measured for the metabolite(s) is below -28‰ based on non-derivatised steroid.

4. Reviewing and evaluating test results:

The following actions should be requested by the Testing Authority in agreement with the Laboratory:

- Isotopic ratios ($^{13}\text{C}/^{12}\text{C}$) of the relevant metabolites should whenever possible be measured each time an elevated parameter of the steroid profile is estimated from the Screening Procedure or Confirmation Procedure and reported to the Testing Authority as having been determined. If the Laboratory does not have the capability to conduct such testing, the Samples are to be securely transferred ensuring the Chain of Custody to another Laboratory with the requisite capability.

- The results of the IRMS analysis and/or of the steroid profile measured by GC/MS shall be used to draw conclusions as to whether a doping violation may have been committed. If the IRMS study does not readily indicate exogenous administration, the result should be reported as “inconclusive” and if necessary further longitudinal studies performed.

- When available, the athlete’s previous tests on record at the Testing Authority should be accessed and the corresponding steroid profile data requested from the relevant Laboratory. These results should be examined and considered together with the existing evidence (longitudinal study).
- If, for any reason, an IRMS analysis cannot be carried out satisfactorily (e.g. insufficient volume of urine, amount of analyte too low to enable a valid measurement) or the examination of previous test results raises suspicions due to unstable profile values, up to three further unannounced tests should be carried out, preferably within a three months period following the report of the suspicious analytical result. There should be a minimum total of three results, other than the abnormal Sample, of either past or post data. A Sample in which the elevated parameter is again measured is to be analysed by IRMS as described above. In difficult cases longer monitoring may be required.

5. Evaluation of longitudinal studies:

In males, the individual T/E values have been shown to vary from their mean value by less than 30% (screening values). In females, a low concentration of some urinary steroids such as epitestosterone and testosterone, close to the limit of detection using current analytical methods occurs. Normal variation of up to 60% may be expected. The individual basal T/E value should be determined from at least three test results, excluding the suspicious result under consideration. The mean, standard deviation and coefficient of variation (expressed in percent) should be calculated for those three basal values. If the suspicious test result, when compared to the basal value using appropriate statistical evaluation is found to be significantly different, that will constitute a proof of the administration of a source of testosterone. It is understood that the basal value may be calculated from previous screening test results. The comparison of screening results and confirmed results is acceptable.

The same reasoning applies to any other parameter of the steroid profile which has been estimated to be in an amount exceeding the ranges of values normally found in humans.

6. Other parameters:

Other parameters such as the ratio of urinary testosterone to Lutenising Hormone (T/LH) and the androsterone to testosterone ratio (A/T) may be used to provide extra information to help determine the use of some substances especially injected testosterone and many of its esters. A high T/LH ratio may be used as ancillary evidence. The A/T ratio which has markedly changed from the “normal” value found for an individual during a longitudinal study may indicate which type of substance has been used. A change to high value can indicate testosterone use and a change to low values may indicate the use of testosterone precursors such as DHEA. However, any administration of testosterone and of its precursors, androstenedione or DHEA will not necessarily alter the excretion of LH and epitestosterone glucuronide.
7. Examples of specific urinary metabolites potentially altered by the administration of “endogenous steroids”;

<table>
<thead>
<tr>
<th>Urinary steroid</th>
<th>Steroid administered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (G)</td>
<td>Testosterone, androstenedione, DHEA</td>
</tr>
<tr>
<td>Epitestosterone (G)</td>
<td>Epitestosterone</td>
</tr>
<tr>
<td>T/E (G)</td>
<td>Testosterone, androstenedione, DHEA</td>
</tr>
<tr>
<td>Androsterone (G)</td>
<td>Testosterone, DHT, androstenedione, DHEA and androstenediol</td>
</tr>
<tr>
<td>Etiocholanolone (G)</td>
<td>Testosterone, androstenedione, DHEA and androstenediol</td>
</tr>
<tr>
<td>DHEA (G) (S)</td>
<td>DHEA</td>
</tr>
<tr>
<td>6α-OH Androstenedione (G)</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>6β-OH Androsterone (G)</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>6β-OH Etiocholanolone(G)</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>6β-OH Epiandrosterone (S)</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>7β-OH DHEA/16α-OH Androsterone (S)</td>
<td>DHEA</td>
</tr>
<tr>
<td>7-OH DHEA, 7 keto DHEA</td>
<td>7 keto DHEA</td>
</tr>
</tbody>
</table>

* G indicates the glucuronide and S indicates sulphate conjugation.

The official text of the technical document Reporting and Evaluation Guidance for Testosterone, Epitestosterone, T/E Ratio and other Endogenous Steroids shall be maintained by WADA and shall be published in English and French. In the event of any conflict between the English and French versions, the English version shall prevail.

8. References:


Horning S., H. Geyer, M. Machnik, W. Schanzer, A. Hilkert and J. Oeßelmann, *Detection of Exogenous testosterone by $^{13}C/^{12}C$ Analysis*, in Recent Advances in Doping Analysis (4),


