Alternative markers for the long-term detection of oral testosterone misuse

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1. Introduction

Testosterone (T) is the principal androgenic anabolic steroid in humans. It is mainly produced in the testis and it is involved in the development of several tissues and processes [1]. T is virtually inactive when administered orally because it is rapidly metabolized mostly to inactive compounds. Its administration as an ester derivative can overcome this limitation. Testosterone undecanoate (TU) is an ester of T which can be used for the treatment of male hypogonadism [2].

Androgenic anabolic steroids can increase lean body mass and strength [3] and can cause several adverse effects for the athlete's health [4]. For these reasons, anabolic androgenic steroids are included in the list of prohibited substances by the World Anti-Doping Agency (WADA) [5]. According to the statistics published by WADA, the category of anabolic agents represents 64.9% of the reported cases by accredited antidoping control laboratories. Among those, 70.3% correspond to T, either as adverse analytical findings [6]. Therefore, the quest for novel analytical approaches for the detection of T misuse is still one of the most important challenges in the doping control field.

Obviously, the main difficulty for the screening of T use is its endogenous nature. Doping control laboratories need to distinguish between T exogenously administered and that endogenously produced. For this purpose, the ratio of urinary testosterone to its isomer epitestosterone (T/E) excreted as glucuronides was proposed in 1983 as the first marker for T misuse [7].

Nowadays, the measure of the T/E is still the gold standard for the screening of T administration. The T/E value can be obtained either by gas chromatography coupled to mass spectrometry (GC–MS) analysis after enzymatic hydrolysis of the urine, and silylation of the steroids [10–12], or by the direct detection of the glucuronides by liquid chromatography tandem mass spectrometry (LC–MS/MS) [13–15]. In recent years, a threshold T/E value of 4 was established by WADA [8]. As a result, samples with a T/E value higher than 4 require a confirmation analysis by isotope ratio monitoring gas chromatography mass spectrometry (GC/C/IRMS) [9]. Nevertheless, many of these confirmation analyses turn out negative, due to, either the athlete had a naturally elevated T/E ratio, or...
the GC/C/IRMS technique was not able to unequivocally prove that part of the T metabolites present in the urine were of exogenous origin.

In spite of the satisfactory results obtained by this approach, the use of the T/E presents some drawbacks. It has been reported that in some cases, after the administration of T to individuals with low basal T/E (often due to genetic factors), the increased concentration of T is not sufficient to raise the T/E above the cut-off value of 4, thus hampering its detection [16,17]. Other scenario where the T/E shows limitations has to do with the short-term detection of testosterone after an oral ingestion. In this case, the T/E value can only be distinguished from the basal level for a few hours (less than 12 h after ingestion), and in some samples the T/E is not affected [18,21]. The detection of the unmodified TU in plasma has been compared with the urinary T/E values but similar detection windows have been obtained [18]. Other analytical strategies have been evaluated for the detection of orally administered T, for instance the detection of the intact administered T esters in hair [19–21]. This approach proved its usefulness in detecting the abuse of T in bodybuilders [20] and in patients taking daily TU [20], however, its application after a single dose administration has not been reported yet. The strategy of detecting a comprehensive panel of urinary steroid by GC/MS, followed by an exhaustive study of alternative ratios has also been evaluated, but none of the proposed markers were found to be better than the classical T/E ratio [22–24].

An alternative and emerging approach is the establishment of individual threshold values. By this strategy, an individual threshold value is calculated considering all available samples for the same athlete. A sample is considered as suspicious if values above this individual threshold are obtained. The use of this approach could overcome the individual and ethnical variations exhibited by the population reference limit [25,26].

Recently, four T metabolites: androsta-1,4-dien-3,17-dione (1,4-AD), androsta-4,6-dien-3,17-dione (4,6-AD), 17β-hydroxy-androsta-4,6-dien-3-one (6-T) and 15-androsten-3,17-dione (15-AD) have been characterized [27], and a adequate method for its quantitation in urine samples have been developed and validated [28]. These metabolites, released after basic treatment of the urine, can be potentially used for the detection of T misuse but its applicability for doping control analysis has not been proved yet.

The goal of this study is to investigate the usefulness of the metabolites released after basic treatment for the detection of orally administered T. Firstly, reference limits for each metabolite and ratio are proposed based on the analysis of samples collected from a healthy population (n = 173). Additionally, samples collected from five volunteers after oral TU intake were analysed. The results are compared with those obtained by the traditional T/E measurement.

2. Experimental

2.1. Chemicals and reagents

Androsta-4,6-dien-3,17-dione (4,6-AD) and 17β-hydroxy-androsta-4,6-dien-3-one (6-T) were obtained from Steraloids Inc. (Newport, USA). Androsta-1,4-dien-3,17-dione (1,4-AD) was purchased from NMI (Pymble, Australia). Methandienone and methyltestosterone were used as internal standard (ISTD) for LC–MS/MS and for GC–MS procedures respectively and testosterone and epitestosterone were obtained from Sigma–Aldrich (St. Louis, MO, USA). The β-glucuronidase preparation (from Escherichia coli type K12) was purchased from Roche Diagnostics (Mannheim, Germany).

Analytical grade potassium carbonate, potassium hydroxide pellets, di-sodium hydrogen phosphate, sodium hydrogen phosphate, tert-butyl-methyl ether, and ammonium iodide were obtained from Merck (Darmstadt, Germany).

Acetonitrile and methanol (LC gradient grade), formic acid, ammonium formate (LC/MS grade) and cyclohexane were purchased from Merck (Darmstadt, Germany). Milli Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Detectabase® columns were obtained from Biochemical Diagnostics Inc (Edgewood, New York, USA).

2.2. LC–MS/MS instrumentation

Selected reaction monitoring (SRM) method was carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UPLC system, Acquity (Waters Associates) for the chromatographic separation. Drying gas as well as nebulising gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. A cone voltage of 25 V, and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 °C and the source temperature to 120 °C.

The LC separation was performed using an Eclipse Plus C18 column (50 × 2.1 mm i.d., 1.8 μm) (Agilent, Palo Alto, CA, USA), at a flow rate of 300 μL/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. A gradient program was used; the percentage of organic solvent was linearly changed as follows: at 0 min, 45%; at 1 min, 45%; at 3.5 min, 65%; at 4.5 min, 95%; at 5 min, 95%; at 5.5 min, 45%; at 8 min, 45%.

Analytes were determined by a SRM method including two transitions for each compound [24]. The most specific transitions (285 > 121 for 1,4-AD, 285 > 149 for 4,6-AD, 287 > 151 for 6-T and 287 > 95 for 15-AD) were selected for quantitative purposes.

2.3. GC–MS instrumentation

GC/MS was carried out on a 6890 N gas chromatograph coupled with a 5975 MSD (Agilent technologies, Palo Alto, CA, USA). The steroids were separated on a HP-Ultra1 cross-linked methyl-silicone column, 16.5 m × 0.2 mm i.d., film thickness 0.11 μm (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a constant pressure of 5 psi. A 2 μL aliquot of the final derivatized extract was injected into the system operated in split mode (split ratio 1:15). The GC temperature is ramped as follows: at 0 min, 180 °C; then 3 °C/min up to 230 °C; then 40 °C/min up to 310 °C; then 3 min to 310 °C.

T and E were determined in SIM mode by monitoring m/z 432 at 13.2 min and 12.3 min respectively. Methyltestosterone used as ISTD was determined by measuring m/z 446 at 14.9 min.

2.4. Sample preparation

The method used for LC–MS/MS determination was based upon the method described and validated elsewhere [25]. Briefly, after addition of 50 μl of ISTD (methandienone at 1 μg/ml), 5 ml of urine were basified by addition of 300 μl of KOH (6 M). The mixture was heated at 60 °C for 15 min, followed by a liquid–liquid extraction with 6 ml of tert-butylmethylether. The sample was centrifuged and the organic layer separated and evaporated. The residue was dissolved into 150 μl of a mixture of water:acetonitrile (50:50, v/v). Finally, 10 μl were directly injected into the LC–MS/MS system.

For GC–MS, after addition of 50 μl of ISTD (methyltestosterone 50 ng/ml), 5 ml of urine were passed through a Detectabase® column, previously conditioned with 2 ml methanol and 2 ml water. The column was then washed with 2 ml water and finally
the analytes were eluted with 2 ml methanol. The methanolic eluate was evaporated under nitrogen stream at 50 °C, reconstituted in 1 ml of sodium phosphate buffer (0.2 M, pH 7), and hydrolysed with 30 μl of β-glucuronidase. The mixture was incubated at 55 °C for 1 h. After cooling to room temperature, 250 μl of a 5% potassium carbonate solution were added to the hydrolysate (pH 9.5). Liquid–liquid extraction was performed by addition of 6 ml tert-butylmethylether. The sample was centrifuged at 3500 rpm, and the organic layer separated, evaporated to dryness under nitrogen stream and dried in a vacuum desiccator over phosphorus pentoxide. The extracts were reconstituted and derivatised with 50 μl of MSTFA:NH₄I:2-Mercaptoethanol (1000:2:6, v/w/v) for 20 min at 60 °C.

2.5. Urine samples

For the administration study, a single oral dose of 120 mg of testosterone undecanoate (Androstan™, three 40-mg capsules; Organon) was given to five healthy male volunteers. Ethical approval for the study had been granted by Comité Ético d’Investigación Clínica of our institute (CEIC-IMAS no. 94/467) and the Spanish Health Ministry (DGEPs no. 95/75). All of the subjects participating in the study gave their written informed consent.

Urine samples were collected before TU administration (three samples) and at 4, 8, 12, 24, 36, 48 and 72 h after TU administration. Aliquots of 50 ml of urine were frozen at −20 °C until analysis.

For the population study, 173 urine samples (134 males and 39 females) were collected. The individuals providing these samples declared not to have taken any prohibited substance.

2.6. Data analysis

The urinary concentrations of the metabolites were corrected to a specific gravity of 1.020 according to WADAS’s technical document on endogenous steroids [8] to reduce variability due to urine dilution.

Urinary concentrations of 1,4-AD, 4,6-AD, 6-T and 15-AD after alkaline treatment were obtained by comparing with a calibration curve. Due to the fact that authentic 15-AD is not commercially available, this steroid was semi-quantified by assuming equal response to 6-T (the analyte with the closest retention time). Ratios between the different metabolites were studied.

The results obtained with the population urine samples (n = 173, 134 males and 39 females) were used to establish the reference limit (RL) values for each metabolite concentration and each ratio. Assuming that the administration of T will increase the concentrations of the metabolites, only the upper limit was used in this study, because suspicious samples will be having a concentration higher than the RL.

The detection time (DT) was defined as the maximum time after TU administration in which the concentration of each marker is above the upper RL obtained in the population studies. The DT obtained for each metabolite and ratio was compared with the DT obtained for T/E.

The program REFVAL was used for the determination of the upper RL [29]. This statically approach was recommended by the Expert Panel on Theory of Reference Values of the International Federation of Clinical Chemistry (IFCC). RL of 97.5% and 99% both with a 95% of confidence interval (CI) were chosen. The descriptive data for the distributions were calculated using the 500 iterative bootstrap method implemented in the REFVAL program. The option in the software for the detection of outliers was not activated.

3. Results

3.1. Concentrations

3.1.1. Testosterone

Urinary concentrations of T after glucuronide hydrolysis were quantified by the conventional GC–MS method applied in routine doping control. The maximum and minimum concentrations obtained in the population study are summarized in Table 1. The 97.5% and 99% RL obtained for T concentration were 89 and 116 ng/ml, respectively.

Basal concentrations below the 97.5% RL were obtained in all volunteers from the TU administration study. Relatively low urinary basal concentrations (between 3 and 20 ng/ml) were detected for volunteers 1 and 3, while higher urinary basal concentrations (between 28 and 51 ng/ml) were detected for volunteers 2, 4 and 5.

Concentrations of T above the 97.5% RL were reported for all volunteers after TU administration with the main exception of volunteer 3, where no rises in T above de 97.5% RL were detected (Fig. 2, Table 2). The highest concentration of T after TU administration was found in the sample collected 4 h after TU administration for volunteer 2 (2456 ng/ml).

The DTs obtained for all volunteers using T concentration as a marker were between 4 and 8 h for all volunteers except for volunteer 3 (Table 2).

3.1.2. Metabolites released after basic treatment

The frequency distributions for 1,4-AD, 4,6-AD, 6-T and 15-AD concentrations of the population samples are shown in Fig. 1. The minimum and maximum concentrations obtained for each metabolite and their 97.5% and 99% RLs with a 95% of CI are summarized in Table 1. Concentrations above the limit of detection of the method [28] (0.006 ng/ml for 1,4-AD, 0.001 ng/ml for 4,6-AD, 0.047 ng/ml for 6-T) were obtained in all samples for all compounds. However, few samples (three for 1,4-AD and one for 4,6-AD, 6-T and 15-AD) were below the limit of quantification of the method i.e. 0.05 ng/ml for 1,4-AD, 15-AD and 6-T and 0.5 ng/ml for 4,6-AD. Concentration values in the sub-ng/ml range (between 0.01 and 1.1 ng/ml) were found for 1,4-AD, while higher concentrations were obtained for 6-T (between 0.20 and 10 ng/ml), 15-AD (between 0.06 and 4.2 ng/ml) and 4,6-AD (between 0.15 and 60 ng/ml).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cmax (ng/ml)</th>
<th>Cmin (ng/ml)</th>
<th>97.5% RL</th>
<th>95% CI</th>
<th>99.0% RL</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>129</td>
<td>1</td>
<td>89</td>
<td>70–111</td>
<td>116</td>
<td>90–129</td>
</tr>
<tr>
<td>E</td>
<td>123</td>
<td>0.5</td>
<td>102</td>
<td>71–124</td>
<td>110</td>
<td>102–123</td>
</tr>
<tr>
<td>1,4-AD</td>
<td>1.1</td>
<td>0.01</td>
<td>0.7</td>
<td>0.5–0.8</td>
<td>0.9</td>
<td>0.7–1.1</td>
</tr>
<tr>
<td>4,6-AD</td>
<td>60</td>
<td>0.15</td>
<td>56</td>
<td>38–60</td>
<td>59.4</td>
<td>54–60</td>
</tr>
<tr>
<td>6-T</td>
<td>10.6</td>
<td>0.02</td>
<td>7.9</td>
<td>6.9–10.3</td>
<td>10.4</td>
<td>7.9–10.6</td>
</tr>
<tr>
<td>15-AD</td>
<td>4.2</td>
<td>0.01</td>
<td>2.7</td>
<td>2.1–3.8</td>
<td>3.8</td>
<td>2.9–4.2</td>
</tr>
</tbody>
</table>

* T and E values were obtained after enzymatic hydrolysis with β-glucuronidase (glucuronide + free fraction).
Basal concentrations in the five volunteers participating in the TU administration study were below the 97.5% RLs for the 1,4-AD (concentrations between 0.18 and 0.5 ng/ml) and 6-T (concentrations between 0.7 and 4.2 ng/ml). In the case of 15-AD and 4,6-AD, most of the volunteers also presented basal concentration values lower the 97.5% RL. However, volunteer 4 had basal concentrations close to the 97.5% RL and in one of the three collected basal samples exceeded this value (Fig. 2).

All four metabolites released after alkaline treatment raised their concentrations above the 97.5% RL after TU administration in all volunteers. The major increases were observed for 1,4-AD and 15-AD where concentrations up to 100 times higher than the 97.5% RL were found in the samples collected 4 h after TU administration. This increase was more moderate for 4,6-AD and 6-T where concentrations 3-4 times higher than the 97.5% RL were obtained in the samples collected 4 h after TU administration (Fig. 2).

The use of 4,6-AD and 6-T concentrations as a markers for TU misuse allows for DTs between 4 and 12 h. However using the concentrations of 1,4-AD and 15-AD, DTs greater than 24 h were in general obtained. Shorter DTs were always observed for volunteer 3 (Table 2).

3.2. Ratios

3.2.1. T/E

The maximum and minimum values found for T/E in the analysis of the population samples were 0.05 and 7.0, respectively. The 97.5% and 99% RLs obtained with this data were 3.5 and 4.6, respectively (Table 3). The results obtained in the population study for T/E ratio started to show the commonly observed bimodal distribution [30,31]. The two maximum modes for the T/E were observed around 0.13 and 1.16 (Fig. 3).
All the volunteers in the TU administration study showed basal T/E values below the 97.5% RL and the threshold established by WADA (4). Three out of the five volunteers had basal T/E values close to the first maximum of the bimodal distribution. Thus, volunteers 1, 3 and 4 had basal T/E values of 0.2, 0.15 and 0.4, respectively. The basal T/E values for the other two volunteers were close to the second maximum of the population distribution (0.9 for volunteer 5 and 1.1 for volunteer 2).

After TU administration, T/E values were more than 50 times higher than the basal ones. The values were normally above the 97.5% RL and the threshold established by WADA (Fig. 4). The main exception was volunteer 3 in which no variation in T/E was observed. The major value in T/E levels after TU administration was observed for volunteer 5 (T/E = 52.9).

Using T/E as marker for T misuse, DTs between 4 and 8 h were obtained for all volunteers with the exception of volunteer 3.

### Table 2
Detection times for the proposed new markers after TU oral administration.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Population data</th>
<th>Detection time (h)</th>
<th>Volunteer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>97.5% RL</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>1,4-AD</td>
<td>0.7</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>4,6-AD</td>
<td>5.6</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>6-T</td>
<td>7.9</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>15-AD</td>
<td>2.7</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>T/E&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>1,4-AD/6T (x10)</td>
<td>2.6</td>
<td>24</td>
<td>All</td>
</tr>
<tr>
<td>15-AD/6-T</td>
<td>1.2</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>1,4-AD/4,6-AD (x100)</td>
<td>3.8</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>15-AD/4,6-AD (x10)</td>
<td>1.8</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>1,4-AD/E (x100)</td>
<td>8.9</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>4,6-AD/E</td>
<td>4.5</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>6-T/E (x10)</td>
<td>6.6</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>15-AD/E (x10)</td>
<td>2.8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> T and T/E values were obtained after enzymatic hydrolysis with β-glucuronidase (glucuronide + free fraction).

### 3.2.2. Metabolites released after alkaline treatment

Twelve ratios can be performed by relation between the four metabolites released after alkaline treatment. The population RLs of each ratio are summarized in Table 3. In order to have more intuitive data, a multiplication factor was applied to some of the ratios (Table 3). Monomodal distributions were obtained for all tested ratios (Fig. 3). The RSD of the population data for all these ratios was also calculated. All ratios have a RSD between 50% and 80%. However, ratios performed between 6-T and 4,6-AD were found to be more constant among the population with a RSD of 25%.

Basal values normally below the 97.5% RLs were obtained for all ratios for all the volunteers from the TU administration study. The main exception for this behaviour was volunteer 2 where the basal values for some ratios like 1,4-AD/6-T were above the 97.5% RL.

After TU administration, the 97.5% RL was exceeded in only four of the twelve tested ratios (1,4-AD/6T, 1,4-AD/4,6-AD, 15-AD/6T, 15-AD/4,6-AD). Therefore, these were the only ratios studied and discussed in this study (Fig. 4). Increases of more than 20 times in the value of these ratios were normally observed after TU administration.
administration (Fig. 4). This rise was observed for all volunteers in all ratios.

The use of 15-AD/4,6-AD and 15-AD/6-T as markers for TU misuse allowed for DTs between 4 and 24 h while longer DTs (between 12 and 48 h) were obtained by the use of 1,4-AD/6-T and 1,4-AD/4,6-AD (Table 2).

### Table 3

<table>
<thead>
<tr>
<th>Ratios</th>
<th>Min</th>
<th>Max</th>
<th>97.5% RL</th>
<th>95% CI</th>
<th>99% RL</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/E</td>
<td>0.05</td>
<td>7.0</td>
<td>3.5</td>
<td>2.5–3.7</td>
<td>4.6</td>
<td>3.5–7.2</td>
</tr>
<tr>
<td>1,4-AD/4,6-AD (100 x)</td>
<td>0.2</td>
<td>4.7</td>
<td>3.8</td>
<td>2.9–4.6</td>
<td>4.6</td>
<td>3.4–4.7</td>
</tr>
<tr>
<td>15-AD/4,6-AD (10 x)</td>
<td>0.05</td>
<td>2.8</td>
<td>1.8</td>
<td>1.2–2.6</td>
<td>2.6</td>
<td>1.6–2.8</td>
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<tr>
<td>6-T/4,6-AD</td>
<td>0.07</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2–0.3</td>
<td>0.3</td>
<td>0.2–0.3</td>
</tr>
<tr>
<td>1,4-AD/6-T (10 x)</td>
<td>0.1</td>
<td>3.9</td>
<td>2.6</td>
<td>2.1–3.3</td>
<td>3.4</td>
<td>2.3–3.9</td>
</tr>
<tr>
<td>4,6-AD/6-T</td>
<td>3.6</td>
<td>14.7</td>
<td>10.9</td>
<td>10.3–14.7</td>
<td>12.4</td>
<td>10.8–14.7</td>
</tr>
<tr>
<td>15-AD/6-T</td>
<td>0.04</td>
<td>2.0</td>
<td>1.1</td>
<td>0.9–1.9</td>
<td>1.9</td>
<td>1.1–2.0</td>
</tr>
<tr>
<td>4,6-AD/1,4-AD (100 x)</td>
<td>0.2</td>
<td>3.8</td>
<td>1.8</td>
<td>1.5–3.4</td>
<td>3.4</td>
<td>1.8–3.8</td>
</tr>
<tr>
<td>6-T/1,4-AD (10)</td>
<td>0.2</td>
<td>6.4</td>
<td>2.9</td>
<td>2.1–6.2</td>
<td>6.2</td>
<td>2.6–6.4</td>
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<tr>
<td>15-AD/1,4-AD</td>
<td>0.4</td>
<td>21.7</td>
<td>10.0</td>
<td>7.8–14.6</td>
<td>14.6</td>
<td>9.3–21.7</td>
</tr>
<tr>
<td>1,4-AD/15-AD (10 x)</td>
<td>0.4</td>
<td>24.1</td>
<td>14.1</td>
<td>8.8–19.6</td>
<td>19.66</td>
<td>24.1</td>
</tr>
<tr>
<td>6-T/15-AD</td>
<td>0.4</td>
<td>22.8</td>
<td>15.6</td>
<td>12.0–17.2</td>
<td>17.3</td>
<td>15.2–22.8</td>
</tr>
<tr>
<td>4,6-AD/15-AD (10)</td>
<td>0.3</td>
<td>20.9</td>
<td>7.7</td>
<td>7.2–11.7</td>
<td>12.3</td>
<td>7.9–20.9</td>
</tr>
<tr>
<td>1,4-AD/E (100 x)</td>
<td>0.03</td>
<td>23.5</td>
<td>8.9</td>
<td>5.2–19.9</td>
<td>20.9</td>
<td>9.3–23.5</td>
</tr>
<tr>
<td>4,6-AD/E</td>
<td>0.02</td>
<td>17.2</td>
<td>4.5</td>
<td>4.1–4.8</td>
<td>8.2</td>
<td>4.5–17.2</td>
</tr>
<tr>
<td>6-T/E (10 x)</td>
<td>0.03</td>
<td>26.5</td>
<td>6.6</td>
<td>5.6–7.9</td>
<td>13.3</td>
<td>6.3–26.5</td>
</tr>
<tr>
<td>15-AD/E (10 x)</td>
<td>0.02</td>
<td>11.0</td>
<td>2.8</td>
<td>2.1–4.0</td>
<td>5.3</td>
<td>2.7–11.0</td>
</tr>
</tbody>
</table>

3.2.3. Metabolites released after alkaline treatment and E

The four ratios between the compounds released after alkaline treatment and E were studied. The population RLs of each ratio are summarized in Table 3. Monomodal distributions were observed for all four ratios (see Supplementary material, Figure A). RSDs between 132% and 176% were obtained.

Basal values below the 97.5% RL were reported for all volunteers from the TU administration study (see Supplementary material, Figure B). After oral TU administration, all of the four ratios within the compounds released after alkaline treatment and E were normally above the 97.5% RL between 4 and 12 h (Table 3). However, using 4,6-AD/E and 6-T/E no values above the 97.5% RL were reported for volunteer 3 (Table 2).

4. Discussion

Population basal levels (n = 173) were used for the establishment of 97.5% and 99% RL with 95% CI of the different markers. The 97.5% RL were chosen in order to establish the DTs. Although a larger number of samples will provide more precise RLs, the T/E threshold of 4 established by WADA for TU misuse is very close to the 97.5% and 99% RLs obtained in the population study described in this paper (3.5 and 4.6, respectively). Therefore, it could be extrapolated that the RLs established in the present study samples are adequate. The RLs obtained for T concentration (89 ng/ml for the 97.5% RL and 116 ng/ml for the 99% RL) are quite far to the RL established by WADA (200 ng/ml). Nevertheless, these values are very similar to those previously reported with the analysis of 3000 samples [31] where a value close of 200 ng/ml was obtained for the 99% RL.

In agreement with previously published data [30,31], T/E exhibited a bimodal distribution. Contrarily, all compounds released after alkaline treatment followed a monomodal distribution (Fig. 1). An additional difference between T and the metabolites released after alkaline treatment is the low basal concentrations found for most of the compounds (typically below 10 ng/ml and in some cases like 1,4-AD below 1 ng/ml). Thus, if it is intended to use these metabolites as markers for TU misuse, high sensitive methods are required.

The study of TU administration samples revealed several of the limitations of using either T concentrations or T/E as markers for oral T administration. On one hand, short DTs (below 8 h) were obtained, and, on the other hand, in the case of volunteer 3, even after administration of 120 mg of TU, neither the concentration nor the T/E ratio increased above the established thresholds (Table 2). The use of 4,6-AD and 6-T concentrations as markers for TU misuse did not improve significantly the retroactivity of T and T/E, meaning that similar DTs were obtained (Figure 3). More promising results were obtained when using 1,4-AD and 15-AD concentrations as markers. In these cases, an important improvement in the retroactivity (between 3 and 6 times compared to T/E) was obtained (Table 2).

The use of concentration ratios can minimize the variations in the sample caused by urinary flow rate. For this reason, several ratios have been proposed specifically as markers for detecting T misuse, although, none of them have improved the discriminatory capability of the T/E ratio for the oral administration of T [18,29]. One of the most critical steps before performing ratios is the selection of the reference compound. Ideally, the reference compound should (i) show remain in altered after the administration, (ii) be excreted as phase II metabolite in the same way of the analyte and (iii) be be detected in the same method of the analyte. In the particular case of T, E satisfies all these conditions and therefore it can be considered as the ideal reference compound for ratios involving T [10].

Contrarily to T and E, the formation of the four studied T metabolites (1,4-AD, 4,6-AD, 6-T and 15-AD) is postulated by release of unknown phase II metabolites because no release of these metabolites after glucuronide hydrolysis or sulphate hydrolysis was reported [28]. Besides, these metabolites and E are determined using different methodologies. Therefore, and taking into account the previously stated considerations, the use of E as reference compound for basic released metabolites is theoretically less suitable than in the case of T.

On the other hand, the four studied T metabolites were quantified using the same analytical method and theoretically, they are formed after realising similar phase II conjugates. However, all the metabolites released after basic treatment showed an increase after T administration. Therefore, none of them was the ideal endogenous reference compound to perform ratios. Among them, 6-T and 4,6-AD exhibited only moderate increases after T administration and, additionally, they showed the lower DTs (Fig. 4). For
these reasons, 6-T and 4,6-AD would be considered as potential endogenous reference compounds when performing ratios. Since no ideal reference compound for performing ratios could be selected, two different approaches were evaluated in this study: (i) performing ratios with E and (ii) performing ratios with 4,6-AD and 6-T.
The eight ratios studied (using E, 6-T and 4,6-AD as reference compound) followed a monomodal distribution (Fig. 3 and Supplementary information Figure A). This fact theoretically represents a valuable advantage compared with the T/E where some of the population has low basal T/E values. Individuals with low basal T/E ratios, will need a substantial increase in T/E ratio before raising the population threshold, so the suspicion of T administration on these samples will be difficult. The use of markers with monomodal distribution can minimize this drawback.

The first tested approach was the use of the ratios within metabolites released after basic treatment and E. A slightly improvement in DTs compared to T/E were observed using 1,4-AD/E and 15-AD/E, whereas worse DTs for 4,6-AD/E and 6-T/E were calculated. The restrospectivity obtained for all these ratios was always smaller than the observed directly by the use of 1,4-AD or 15-AD concentrations. The high RSD showed for these ratios (higher than 130%) indicates a high variability among the population values which entails high RLs and decreases the retrospectivity. This fact hampers the use of ratios between the studied metabolites and E for the detection of oral T misuse. More promising results were found by performing ratios with 4,6-AD and 6-T. Using 1,4-AD/6-T and 1,4-AD/4,6-AD a significant improvement in retrospectivity (between 3 and 6 times compared to T/E) were found (Table 2 and Fig. 4). These results are similar to those obtained with the concentration of 1,4-AD (Table 2). However, as stated above the application of ratios is always preferred because it can minimize the variations in the sample produced by urinary dilution. Therefore, the establishment of 1,4-AD/6-T and 1,4-AD/4,6-AD can be a valuable alternatives for the detection of oral T misuse. This fact is illustrated in Fig. 5 where the chromatograms obtained for volunteer 4 using T/E and 1,4-AD/6-T before and after oral T administration are shown. No significant differences were observed for T/E between the chromatograms obtained before and 24 h after TU administration. However, using 1,4-AD/6-T even 24 h after TU administration clear differences between both chromatograms were found.

The use of ratios involving 15-AD (15-AD/4,6-AD and 15-AD/6-T) also allowed for a two fold increase in the DT when compared to those obtained with T/E. Nevertheless, their DT are shorter than those obtained with 1,4-AD/6-T and 1,4-AD/4,6-AD.

It is interesting to comment the case of volunteer 3. T/E ratio was no useful in this volunteer to detect TU misuse since no rises above the RL were obtained. This behaviour can be explained by with the low basal T/E for this volunteer, related to the deletion polymorphism in the gene coding for UGT2B17 [32]. The gene UGT2B17 is associated with T glucuronide levels in urine. Subjects with such deletion in this gene could have a T/E lower than 0.4 [33,34]. Although this deletion can contribute to explain the absence of variation in T/E values after TU administration, it has to be taken into account that volunteers 1 and 4 also have low basal T/E values and the detection of the misuse was still possible. Although the nature of the conjugate released after basic treatment is still unknown, it was demonstrated that it is not a glucuronide [27]. Therefore, the use of these metabolites can minimize those
limitations associated with the glucuronidation step. This fact is demonstrated with volunteer 3 where the use of the metabolites released after alkaline treatment and ratios between these compounds, allowed for the detection of T misuse up to 12 h in the best cases.

The usefulness of these markers has been demonstrated for oral T misuse. It will be of interest to evaluate the usefulness of these markers for the detection of the administration of other forms of T i.e. topical and intramuscular administration. For this purpose, the RLs established in the present study could be used. Samples collected after the administration of testosterone through different routes should be analysed using the same approach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2011.07.005.

References


