Accepted Manuscript

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PII: S0960-0760(16)30178-9
DOI: <http://dx.doi.org/doi:10.1016/j.jsbmb.2016.06.006>
Reference: SBMB 4744

To appear in: *Journal of Steroid Biochemistry & Molecular Biology*

Received date: 20-11-2015
Revised date: 11-5-2016
Accepted date: 17-6-2016

Please cite this article as: Aristotelis Kotronoulas, Àlex Gomez-Gomez, Jordi Segura, Rosa Ventura, Jesús Joglar, Oscar J. Pozo, Evaluation of two glucuronides resistant to enzymatic hydrolysis as markers of testosterone oral administration, *Journal of Steroid Biochemistry and Molecular Biology* <http://dx.doi.org/10.1016/j.jsbmb.2016.06.006>

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Evaluation of two glucuronides resistant to enzymatic hydrolysis as markers of testosterone oral administration

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Graphical abstract

Highlights

- T metabolites resistant to hydrolysis are evaluated for the screening of oral administration
- Four markers including these metabolites increased the detectability of the administration
- Prolonged detection of T administration even when urinary TG fails to detect the administration
- High intra-individual stability for all evaluated markers

Abstract

Testosterone (T) has traditionally been the most commonly reported doping agent by doping control laboratories. The screening of T misuse is performed by the quantification of six endogenous androgenic steroids and the ratio T/E included in the Athletes Biological Passport (ABP). The inclusion of additional metabolites can improve the screening capabilities of ABP. In this study, the potential of 3 α -glucuronide-6 β -hydroxyandrosterone (6OH-Andros3G) and 3 α -glucuronide-6 β -hydroxyetiocholanolone (6OH-Etio3G) as markers of T oral administration was evaluated. These glucuronides have been shown to be resistant to enzymatic hydrolysis and their quantification by means of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was reported as the only way to obtain feasible results. Urine samples were collected from five volunteers before and after the oral administration of 40 mg of T undecanoate and were analyzed by a LC-MS/MS method recently developed. Concentration of 6OH-Andros3G and 6OH-Etio3G compounds and those of the glucuronides of T (TG), epitestosterone (EG), androsterone and etiocholanolone were established and different concentration ratios were calculated. The detection windows (DWs) for the T administration obtained by each selected ratio were compared to the one of TG/EG. The results showed that four out of the nine tested markers presented DWs much larger for all volunteers than those obtained by the World Anti Doping Association established T/E marker or other alternative markers. The 6OH-Andros3G/EG, 6OH-Etio3G/EG, 6OH-Andros3G/TG and 6OH-Etio3G/TG markers were able to identify the T abuse up to 96 h after the administration, extending our detection capability for the misuse up to 84h more than the classic marker. The importance of these markers was also highlighted by their prolonged capacity to detect the T misuse in

the case of one volunteer who's TG/EG barely exceeded his individual threshold. As a consequence, the four markers presented in this study seem to have an exceptional potential as biomarkers of T oral administration.

Abbreviations

3OH-Andros6G: 6 β -glucuronide-3 α -hydroxyandrosterone

6OH-Andros3G: 3 α -glucuronide-6 β -hydroxyandrosterone

6OH-Etio3G: 3 α -glucuronide-6 β -hydroxyetiocholanolone

ABP: athlete biological passport

Andros: androsterone

AndrosG: Andros glucuronide

d₃-EG: d₃-epitestosterone glucuronide

d₃-TG: d₃-testosterone glucuronide

d₄-AndrosG: d₄-Androsterone glucuronide

DWs: detection windows

E: epitestosterone

EG: epitestosterone glucuronide

Etio: etiocholanolone

EtioG: Etio glucuronide

GC-MS(/MS): gas-chromatography (tandem) mass spectrometry

LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry

T: testosterone

TG: testosterone glucuronide

WADA: World Anti-Doping Agency

Keywords: testosterone; phase II metabolites; LC-MS/MS; doping control; steroid profile

1. Introduction

Testosterone (T) has traditionally been the most commonly doping agent reported by the doping control laboratories [1]. The current methodology used by these laboratories for the detection of T misuse is based on the pioneer studies performed in the early 80's by Donike et al. [2]. These studies revealed that the ratio between the glucuronides of T and its epimer epitestosterone (E) is significantly affected by T exogenous administration. During the years, even though the T/E remained the most important marker, more T metabolites were reported as markers for the misuse of T or other endogenous androgenic steroids [3-5]. Today, a steroid profile composed by seven markers has been established by the World Anti-Doping Agency (WADA) in order to improve the screening for T misuse. These markers include the urinary concentrations of T, E, androsterone (Andros), etiocholanolone (Etio), 5α -androstane- $3\alpha,17\beta$ -diol and 5β -androstane- $3\alpha,17\beta$ -diol as well as the T/E, measured after hydrolysis with β -glucuronidase, and they are reported by the doping control laboratories to WADA for evaluation of any atypical findings [6].

The establishment of universal criteria for the report of T misuse was questioned when several studies reported that the values of these seven markers is severely affected by certain genetic differences among populations [7-9]. However, the steroid profile parameters were shown to be relatively stable within an individual [10] and for this reason Sottas et al. [11] proposed a novel statistical tool that permitted the continuous and systematic evaluation of the seven markers of the steroid profile individually for all athletes. This approach, based on the Bayesian adaptive model, combined population-based with individual-based information in order to generate the upper and lower limits for each

marker in every individual [12]. In these models, as the individual-based measurements sequentially grow in number, the individual thresholds for each biomarker are recalculated and adapted to the athlete's individual values. In this way, individual thresholds of each marker concentration or value can be established for every athlete and unexpected changes in his/her steroid profile can be detected. In order to register the steroid profile data for each athlete individually WADA has recently introduced the athlete steroidal passport as a module of the athlete biological passport (ABP) [13].

In any case, the steroid profile should not be seen as a static tool since the inclusion of additional metabolites has been reported as an efficient way to improve the screening capabilities of the ABP [14,15]. In fact, additional markers can complete the screening capability of the ABP and expand the detection windows (DWs) of the T misuse. Currently, the quantification of the glucuronides of the steroid profile is based on a gas-chromatography (tandem) mass spectrometry (GC-MS(/MS)) procedure that includes various laborious steps, such as enzymatic hydrolysis of the sample and its derivatization [6]. However, the investigation using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is becoming more popular due to its advantages over the classic GC-MS(/MS) procedure [16]. The use of the LC-MS/MS allows for the direct analysis of intact phase II metabolites [17-19], avoiding the time consuming and laborious steps of enzymatic hydrolysis and derivatization needed for the GC-MS analysis. Additionally, the coupling of LC with versatile and sensitive MS analyzers allowed for the reevaluation of the T metabolome [20-22] and led to the identification of many unreported metabolites of endogenous steroids [20,23-26].

In a previous study conducted by our laboratory [24], the application of a LC-MS/MS method for the open detection of steroid glucuronides in urine samples revealed the

presence of two glucuronides that increased after oral administration of T undecanoate and greatly resisted the enzymatic hydrolysis of the urine samples by β -glucuronidase [24]. These metabolites were subsequently identified as the 3α -glucuronide- 6β -hydroxyandrosterone (6OH-Andros3G) and the 3α -glucuronide- 6β -hydroxyetiocholanolone (6OH-Etio3G) [27] and a quantitative UHPLC-MS/MS method was recently developed and validated for their proper quantification [28]. This quantitative method also included the main glucuronides that compose the steroid profile, namely the T glucuronide (TG), E glucuronide (EG), Andros glucuronide (AndrosG) and Etio glucuronide (EtioG) [28]. The use of LC-MS based approaches was shown to be the most reliable strategy for the correct quantification of 6OH-Andros3G and 6OH-Etio3G, since results obtained by common GC-MS(/MS) methods are critically affected by the presence of matrix interferences [28].

The aim of this study was to evaluate the usefulness of these resistant to enzymatic hydrolysis glucuronides as biomarkers of T administration. For this reason, urine samples collected before and after the oral administration of T undecanoate to $n=5$ volunteers were analyzed by the validated UHPLC-MS/MS method. The ratios of 6OH-Andros3G and 6OH-Etio3G metabolites with the EG and the TG were calculated and compared with the TG/EG.

2. Experimental

2.1. Chemicals and reagents

6OH-Andros3G, 6OH-Etio3G and 6β -glucuronide- 3α -hydroxyandrosterone (**3OH-Andros6G**, used as internal standard, ISTD) were synthesized as previously

described [27]. TG, EG, d₃-testosterone glucuronide (d₃-TG) and d₃-epitestosterone glucuronide (d₃-EG) were purchased from the Australian National Measurement Institute (Pymble, Australia). AndrosG and EtioG were obtained from Steraloids Inc. (Newport, RI, USA). d₄-Androsterone glucuronide (d₄-AndrosG) was obtained from Orphachem (Saint-Beauzive, France). Methanol (HPLC gradient grade), formic acid and ammonium formate (LC/MS grade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.2. LC-MS/MS instrumentation

Chromatographic separation was performed by using an Acquity UPLC instrument (Waters, Milford, Massachusetts, USA). The LC system was equipped with an Acquity UPLC[®] BEH C18 1.7 μ m 2.1x100 mm column (Waters). The injection volume was 10 μ L and the flow rate 0.3 mL/min. Water (A) and methanol (B) both with 0.01 % HCOOH and 1 mM ammonium formate were selected as mobile phase solvents. During the gradient program used, the percentage of organic solvent changed as following: 0 min, 20%; 9 min, 65%; 10 min 95%; 11 min; 95%; 12 min 20%; 14 min; 20%.

The detection was performed with a triple quadrupole (Quattro Premier XE, Waters Associates) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI). The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. Nitrogen was used as drying and nebulising gas. A capillary voltage of 3.0 kV was used in positive ionization mode. The nitrogen desolvation temperature was set to 450 °C and the source temperature to 120 °C. The selected reaction monitoring (SRM) method used for the quantification of all analytes was the same used in a previous study [28] and is summarized in Table 1.

2.3. Sample preparation

After the addition of 20 μ L of the internal standard (ISTD) mix solution (2.5 μ g/mL 6OH-Andros-6-Gluc, 1.25 μ g/mL d3-TG; 1.25 μ g/mL d3-EG and 25 μ g/mL d4-Andros-Gluc), urine samples (0.5 mL) were passed through a C18 cartridge (Sep-Pak Vac RC, 100 mg, Waters Associates), previously conditioned with 1 mL of methanol and 1 mL of water. The column was then washed with 2 mL of water, and the analytes were eluted with 2 mL of methanol. The eluate was evaporated to dryness under a nitrogen stream in a water bath at 50 °C. Then, the dry extract was reconstituted in 150 μ L of water and methanol (9:1, v/v) mixture and 10 μ L were directly injected into the LC-MS/MS system.

2.4. Urine samples

A single oral dose of 120 mg of testosterone undecanoate (Androxon™, three 40 mg capsules; Organon) was given to five healthy male volunteers. All of the subjects participating in the study gave their written informed consent. Three urine samples were collected before TU administration from all volunteers (-48 h, -24 h and 0h). After the administration, the urine samples were collected at 4, 8, 12, 24, 36, 48, 72, 96 and 144 h. Aliquots of 50 mL of urine were frozen at -20 °C until analysis.

Ethical approval for the study had been granted by Comitè Ètic d'Investigació Clínica of our institute (CEIC-IMAS no. 94/467) and the Spanish Health Ministry (DGFPS no. 95/75).

2.5. Calibration curves and quality control samples

The quantification of the analytes was based on a previously developed and validated method [28]. Calibration curves were prepared by adding the appropriate volumes of working solutions of all analytes and 20 μ L of the ISTD mix solution to test tubes. The solvent was evaporated under N₂ (25°C, 10–15 psi) and 0.5 mL of ultra-pure water was added. Final concentrations for the calibration curves were 50, 100, 250 ng/mL for the 6OH-Andros3G, 10, 20, 50 ng/mL for the 6OH-Etio3G, 5, 20, 50 ng/mL for the TG and EG and 250, 1000, 2500 ng/mL for the AndrosG and EtioG.

For the quality control (QC) samples, aliquots (0.5 mL) of a spot urine sample collected from one volunteer (different to the volunteers that participated to the T administration experiment) were prepared and immediately frozen at -20 °C until each day of analysis. Two QC samples were added to each batch of analysis in order to monitor the inter-day deviation of the measurements. The analysis was considered accurate if the calculated concentration for every analyte in the QC samples was within the limits provided by the equation: $QC_{limits} = Average \pm 3 * Standard\ Deviation$ (SD, for a 99.7% of confidence).

2.6 Data analysis

Quantification of all metabolites was performed using MassLynx software (version 4.1) from Waters.

The relative standard deviation (RSD) was calculated as the percentage of the standard deviation over the average value.

In order to evaluate the usefulness of the 6OH-Andros3G and 6OH-Etio3G, the DWs of the potential biomarkers were calculated using a modification of Bayesian adaptive model [11,12]. Briefly, the basal samples (n=3) collected before the administration for each volunteer were considered as a previously individual longitudinal data for each volunteer. The low number of basal samples advised against the strict use of the ABP software for the establishment of the individual threshold. Thus, a previously reported approach [29] [refs] which does not take into account the population values was selected for this purpose. The individual threshold value for each biomarker was calculated by the following equation: $Threshold = (AB) + 3*(SD_{basal})$, where (AB) is the analyte's average concentration of the basal samples and (SD_{basal}) is the standard deviation between the concentrations of the basal samples. The DW for each marker was defined as the maximum time after the administration in which values exceeded the respective threshold.

2.7 Studied markers and evaluation criteria

The concentration ratio between TG and EG (TG/EG) and the nine possible ratios that involve the concentrations of either 6OH-Andros3G or 6OH-Etio3G as dividend (6OH-Andros3G/EG, 6OH-Etio3G/EG, 6OH-Andros3G/TG, 6OH-Etio3G/TG, 6OH-Andros3G/AndrosG, 6OH-Etio3G/AndrosG, 6OH-Andros3G/EtioG, 6OH-Etio3G/EtioG and 6OH-Andros3G/6OH-Etio3G) were calculated for all samples collected before and after the T administration. For the evaluation, any marker that presented DW greater than that offered by the TG/EG in all volunteers was considered eligible.

3. Results

3.1 Selection of markers

The DWs provided by TG/EG for each volunteer were used as reference for the evaluation of the novel markers. Moreover, among the nine tested markers, ratios of 6OH-Andros3G and 6OH-Etio3G with AndrosG and EtioG presented DWs that were similar or lower than the ones obtained by the TG/EG whereas the 6OH-Andros3G/6OH-Etio3G marker did not present any diagnostic power at all. According to the evaluation criteria, 6OH-Andros3G/EG, 6OH-Etio3G/EG, 6OH-Andros3G/TG and 6OH-Etio3G/TG showed acceptable DWs (Table 2) and their behavior pattern was further analyzed.

3.2 Basal levels

The average basal values of the TG/EG, 6OH-Andros3G/EG, 6OH-Etio3G/EG, 6OH-Andros3G/TG and 6OH-Etio3G/TG are summarized in Table 3. As shown, two of the volunteers presented TG/EG values that were greater than 1 (volunteers 2 and 5) while the rest of the volunteers presented significantly lower TG/EG values. The low TG/EG values obtained for volunteers 1, 3 and 4 were mostly related to low concentrations of TG rather than high concentrations of EG (data not shown). In addition, the values of TG/EG were in line to those obtained for the same samples in the previous studies where the GC-MS(/MS) procedure for the calculation of the T/E was followed [14,30].

The analysis of the basal samples revealed that the average concentrations of the 6OH-Andros3G and 6OH-Etio3G (data not shown) were within the expected ranges described for these compounds [28]. As shown in Table 3, the average basal values of volunteers 1-4 for the 6OH-Andros3G and 6OH-Etio3G ratios with the EG were respectively in the range of 1.25 to 2.23 (6OH-Andros3G/EG) and of 0.15 to 0.41 (6OH-Etio3G/EG). Volunteer 5 presented around four fold higher values for the two markers (5.46 and 0.47 respectively). Moreover, the basal values observed in the case of the 6OH-Andros3G/TG and 6OH-Etio3G/TG markers ranged between 1.31 to 4.28 and 0.11 to 0.47 respectively for volunteers 1, 2, 4 and 5. Higher values of the two markers were calculated for volunteer 3 (9.13 for 6OH-Andros3G/TG and 2.14 for 6OH-Etio3G/TG).

The calculated RSDs, whose values are related to the intra-individual stability of a marker, were shown to be relatively low for all tested markers. The only exception for this behaviour was the RSDs obtained for the average values of 6OH-Etio3G/EG and 6OH-Etio3G/TG for volunteer 1 and 2.

3.3 TG/EG marker after the oral T administration

The changes of the TG/EG after T administration for volunteer 1 and 3 are plotted in Figure 1. As shown, a high increase of the TG/EG was observed in the first 4 h for volunteer 1 (Figure 1A) that reached approximately 20 folds over his individual threshold. Similar increases were also observed for volunteers 2, 4 and 5. After that, the levels of the TG/EG returned to their basal values allowing for the detection of the T misuse from 12 h to 48 h for these volunteers (Table 2). However, in the case of volunteer 3 (Figure 1B), the levels of the TG/EG only exceeded the individual threshold approximately 0.5 folds in the sample obtained 4 h after the administration of T. For all the other samples collected after

the administration for this volunteer, the TG/EG levels were maintained below his individual threshold.

3.3 Markers involving the 6OH-Andros3G and 6OH-Etio3G metabolites

The changes of the 6OH-Andros3G/EG and 6OH-Etio3G/EG values after the oral administration of T undecanoate for volunteers 1 and 3 are shown in Figure 2. For all volunteers, including volunteers 2, 4 and 5, both markers reached their maximum value 4 h after the administration and then started to decrease. The DWs obtained by the 6OH-Andros3G/EG marker extended from 24 h to 72 h for all volunteers (Table 2), showing an increase in the detection time of T administration up to 60 h (volunteer 1) in comparison to the TG/EG. In the case of the 6OH-Etio3G/EG marker, DWs of 48 h were obtained for volunteers 1-4 while for volunteer 5 the detection time was 96 h after T administration (Table 2). These DWs calculated by the use of the 6OH-Etio3G/EG marker were always larger than the ones offered by the TG/EG.

The alterations in the profile the 6OH-Andros3G/TG and 6OH-Etio3G/TG markers after T administration can be observed in Figure 3 for volunteers 1 and 3. In this case, the maximum values of the markers were reached in 8 to 12 h after the administration for all volunteers. As shown in Table 2, the DWs obtained by the two markers extended between 36 h and 96 h for all volunteers. The 6OH-Andros3G/TG marker prolonged the detection up to 84 h more than the TG/EG (volunteer 1) and the 6OH-Etio3G/TG marker increased the DWs over the TG/EG up to 48 h (volunteer 4).

4. Discussion

The T/E has been extensively used as the main marker for the detection of T misuse and it is the most important among the seven markers included in the steroid profile established by WADA [6]. Apart from the small intra-individual variations of its basal values, even after severe physical exercise, and their stability during circadian, menstrual or annual cycles, its importance has been broadly demonstrated in several studies due to the high sensitivity that this marker exhibits to the external administration of T in most of the cases [10]. The UHPLC-MS/MS method used in this study permits the direct quantification of the intact glucuronides of both T and E (TG and EG respectively) instead of their indirect measurement by means of enzymatic hydrolysis and GC-MS(/MS) analysis that is currently applied in the anti-doping laboratories. However, the values of the TG/EG calculated by the UHPLC-MS/MS method have been shown to be highly correlated to T/E values obtained by the standard GC-MS(/MS) procedure and the acquired measurements for these markers are comparable [28]. Thus, the behavioral patterns of the T/E described in literature [10] can be directly linked to the TG/EG.

The analysis of the basal samples (Table 3) showed that TG/EG values for all volunteers were between the reference range for male Caucasian population [31]. The TG/EG values for volunteers 1, 3 and 4 (0.32, 0.20 and 0.50 respectively) seemed to be in agreement with a deletion in the UGT2B17 gene [32,33].

Contrary to the metabolites that compose the steroid profile, no previous analysis of potential markers that involve the 6OH-Andros3G and 6OH-Etio3G has been previously performed. In our study, eight ratios between either the 6OH-Andros3G or 6OH-Etio3G and TG, EG, AndrosG and EtioG as well as the 6OH-Andros3G/6OH-Etio3G were

evaluated. However, only four of them (6OH-Andros3G/EG, 6OH-Etio3G/EG, 6OH-Andros3G/TG and 6OH-Etio3G/TG) gave eligible results and their behavior was further analyzed.

The average basal values of all evaluated markers normally showed low RSDs indicating consistency within the same volunteer. In general, the small variability of a marker's basal values is crucial for its inclusion in the ABP since the determination of the individual thresholds depends on the standard deviation of the marker. Hence, the larger the SD of a marker among basal samples of the same individual, the higher the individual threshold of the marker and the detection of the misuse will be more difficult. For this reason, markers that present high intra-individual stability are preferred. In this particular case of the oral T administration where the maximum values exceeded many folds the individual threshold values in post-administration samples (Figure 2 and 3), the low values of the RSD obtained for the markers could be considered to have a relative limited impact on the evaluation. However, the intra-individual stability of these markers, suggested by our experiments, can be important for the detection of lower doses of oral administration or for the detection of other types of T administration, e.g. intramuscular or transdermal.

After the oral administration of T undecanoate, TG/EG gave DWs for the detection of T that were between 12 h to 48 h for volunteers 1, 2, 4 and 5 (Table 2). As expected, these DWs were greater than previously reported detection times based on population reference limits [14,34]. However, they can be considered within the expected ranges of detection when individual limits are used. The only exception for this behavior were the TG/EG values obtained for volunteer 3 (Figure 1B). In this case, the TG/EG levels for this volunteer were maintained below the threshold value for almost all the analyzed post-administration samples.

The DWs obtained for the four tested markers were compared to those of the classic TG/EG in order to evaluate their usefulness for the detection of oral T administration. The results showed that 6OH-Andros3G/EG and 6OH-Etio3G/EG have a big potential for the detection of oral T misuse since both of them provided DWs that exceeded for many hours those obtained by the classic TG/EG marker (Table 2). However, DWs larger than those of the 6OH-Andros3G/EG and 6OH-Etio3G/EG were the ones provided by 6OH-Andros3G/TG and 6OH-Etio3G/TG which prolonged the detection of T administration up to 84 h more than the TG/EG (Table 2). A negative feedback in the production of testosterone can be the most feasible reason for the larger DWs obtained by these ratios.

In all cases, the detection times provided by the four novel markers analyzed in this study were apparently longer than those obtained in our previous work where the potential of several cysteinyl conjugate T metabolites [24] released after alkaline treatment of samples [25] were evaluated for the same urine samples [14]. However, these results were calculated by population, which normally provide shorter DWs than the individual thresholds used in the ABP. Similar circumstances characterize other studies that explore alternative markers for T misuse [30,35], suggesting the reevaluation of their results with individual thresholds.

Finally, it has been demonstrated that a certain genetic discrepancy among individuals can decrease the glucuronization rate of T [32,33] and can lead to low T/E values even after T administration [8]. Although the use of individual limits has decreased the number of falsely reported atypical findings related to genetic differences, the evaluation of novel markers should examine their possible effect on the detection of T misuse e.g. among populations with different steroid glucuronidation profile, such as the Caucasian and Asian [33]. In our study, all tested markers were capable of detecting the T

administration independently of their average basal values and the possible genetic discrepancies among individuals. In addition, it has to be remarked that, in the case of volunteer 3, the detection of T administration by the TG/EG was possible only in the sample collected 4h after the administration. However, all markers evaluated in this study were able to detect the T administration for many more hours, exhibiting a significant advantage over the classic TG/EG for the screening of T oral administration.

5. Conclusions

The potential of two glucuronoconjugated metabolites of T (6OH-Andros3G and 6OH-Etio3G) that are resistant to enzymatic hydrolysis as biomarkers of oral T misuse was evaluated after one single dose of T undecanoate. The concentration ratios of these compounds with the TG, EG, AndrosG and EtioG were calculated after the oral administration of T undecanoate and the respective DWs for the detection of T misuse offered by each marker were compared to the TG/EG. The results showed that the 6OH-Andros3G/EG, 6OH-Etio3G/EG, 6OH-Andros3G/TG and 6OH-Etio3G/TG presented much larger DWs than those obtained by the established T/E marker for all volunteers, extending our detection capability up to 84h more than the classic marker. In addition, the importance of these markers was highlighted by their capacity to prolong the detection of T misuse in the case of one volunteer who's TG/EG barely exceeded his individual threshold. Altogether, despite the limited number of volunteers, these results suggest the inclusion of these markers in the ABP as complement of the current steroid profile and open up the road for more extended studies involving a larger number of volunteers.. However, more studies should be conducted before this inclusion. The usefulness of these markers for the detection

of other administration routes and the occurrence of possible genetic discrepancies among ethnic populations should be investigated.

Acknowledgments

Grants by World Anti-Doping Agency (14A29OP) and support by the Catalan Government (2014 SGR 692) are gratefully acknowledged. Spanish Health National System is acknowledged for O. J. Pozo contract (MS10/00576).

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Figure 1. The classic T/E marker shows limited DW after T administration and even fails to detect it for volunteer 3: TG/EG values for volunteer 1 (A) and volunteer 3 (B) during time before and after the administration of T undecanoate. Threshold value obtained as reported in Experimental section

Figure 2. 6OH-Andros3G/EG and 6OH-Etio3G/EG markers increase the DW after oral T administration even for volunteer 3: 6OH-Andros3G/EG (C and D) and 6OH-Etio3G/EG (E and F) values for volunteers 1 and 3 during time before and after the administration of T undecanoate. Threshold value obtained as reported in Experimental section

Figure 3. 6OH-Andros3G/TG and 6OH-Etio3G/TG markers increase the DW after oral T administration even for volunteer 3: 6OH-Andros3G/TG (G and H) and 6OH-Etio3G/TG (I and J) values for volunteers 1 and 3 during time before and after the administration of T undecanoate. Threshold value obtained as reported in Experimental section.

Table 1. SRM methods for the detection of steroid glucuronides (*transitions used for quantification)

Analyte	Retention time (min)	Precursor ion (m/z)	Cone (V)	Collision energy (eV)	Product ion (m/z)
6OH-Andros3G	5.5	500	15	40	81*
				30	271
6OH-Etio3G	5.1	500	15	40	81*
				30	271
TG	7.5	465	25	30	97*
				20	289
EG	8.9	465	15	10	289*
				35	97
AndrosG	9.7	484	25	30	141*
				10	291
EtioG	9.5	484	25	30	141*
				10	291
Internal Standards					
3OH-Andros6G	4.9	500	15	40	81*
d3-TG	7.5	468	25	30	97*
d3-EG	8.9	468	15	10	292*
d4-AndrosG	9.7	488	25	30	141*

Table 2: Detection windows (DWs) in hours obtained for the selected markers

	Vol 1	Vol 2	Vol 3	Vol 4	Vol 5
<i>TG/EG</i>	12	36	4	24	48
<i>6OH-Andros3G/EG</i>	72	48	24	36	72
<i>6OH-Etio3G/EG</i>	48	48	48	48	96
<i>6OH-Andros3G/TG</i>	96	48	36	36	96
<i>6OH-Etio3G/TG</i>	48	48	48	72	96

Table 3: Average basal values of the selected markers for all volunteers. In parenthesis the calculated RSD for each average value.

	Vol 1	Vol 2	Vol 3	Vol 4	Vol 5
<i>TG/EG</i>	0.32 (6%)	1.70 (2%)	0.20 (5%)	0.50 (2%)	1.27 (1%)
<i>6OH-Andros3G/EG</i>	1.25 (10%)	2.23 (8%)	1.77 (20%)	1.49 (6%)	5.46 (15%)
<i>6OH-Etio3G/EG</i>	0.15 (27%)	0.19 (32%)	0.41 (12%)	0.18 (6%)	0.47 (9%)
<i>6OH-Andros3G/TG</i>	3.86 (12%)	1.31 (9%)	9.13 (14%)	3.01 (7%)	4.28 (5%)
<i>6OH-Etio3G/TG</i>	0.47 (19%)	0.11 (27%)	2.14 (10%)	0.37 (5%)	0.37 (3%)