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

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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 Athlete Biological Passport (ABP). The inclusion of additional metabolites can improve the screening capabilities of ABP. In this study, the potential of 3α-glucuronide-6β-hydroxyandrosterone (6OH-Andros3G) and 3α-glucuronide-6β-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 Agency 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 84 h 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 whose 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.

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 epitestoterone (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

Abbreviations: 3OH-Andros6G, 6β-glucuronide-3α-hydroxyandrosterone; 6OH-Andros3G, 3α-glucuronide-6β-hydroxyandrosterone; 6OH-Etio3G, 3α-glucuronide-6β-hydroxyetiocholanolone; ABP, athlete biological passport; Andros, androsterone; AndrosG, andros glucuronide; d_3-EG, d_3-epitestosterone glucuronide; d_3-TG, d_3-testosterone glucuronide; d_4-AndrosG, d_4-androsterone glucuronide; DWs, detection windows; E, epitestoterone; EG, epitestoterone 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.

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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-androstan-3α,17β-diol and 5β-androstan-3α,17β-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, d_3-testosterone glucuronide (d_3-TG) and d_3-epitestosterone glucuronide (d_3-EG) were purchased from the Australian National Measurement Institute (Pymble, Australia). AndrosG and EtioG were obtained from Steraloids Inc. (Newport, RI, USA). d_4-Androsterone glucuronide (d_4-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 μm 2.1 × 100 mm column (Waters). The injection volume was 10 μL 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 μL of the internal standard (ISTD) mix solution (2.5 μg/mL 6OH-Andros-6G, 1.25 μg/mL d3-TG; 1.25 μg/mL d3-EG and 25 μg/mL d4-AndrosG), 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 μL of water and methanol (9:1, v/v) mixture and 10 μL were directly injected into the LC–MS/MS system.