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Ultra high performance liquid chromatography tandem mass spectrometric detection of glucuronides resistant to enzymatic hydrolysis: Implications to doping control analysis

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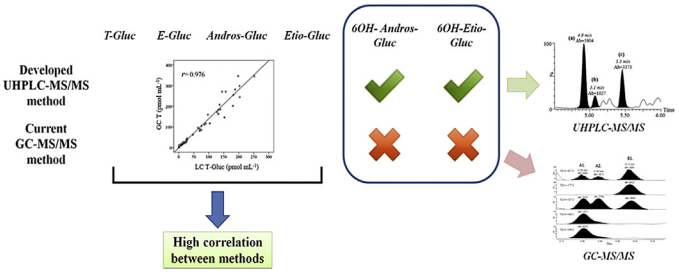
HIGHLIGHTS

- Controversial behavior of 6OH-Andros-3-Gluc and 6OH-Etio-3-Gluc was evaluated.
- UHPLC–MS/MS method for the quantification of resistant glucuronides was validated.
- Only LC–MS methods can properly quantify the 6OH-Andros-3-Gluc and 6OH-Etio-3-Gluc.
- Common GC–MS methods misidentify these metabolites due to matrix interferences.
- These metabolites seem suitable markers for the screening of testosterone misuse.

GRAPHICAL ABSTRACT



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ABSTRACT

Controversial results have been reported in the literature regarding the behavior of two testosterone (T) metabolites (3α-glucuronide-6β-hydroxyandrosterone and 3α-glucuronide-6β-hydroxyetiocholanolone) excreted after T administration. Due to their potential as biomarkers of T misuse, a UHPLC–MS/MS method for the direct quantification of these glucuronides was developed and validated. In addition, the main phase II metabolites of T that compose the steroid profile used for doping control purposes (glucuronides of T, epitestosterone, androsterone and etiocholanolone) were included. The method was found to be linear and with suitable LODs and LOQs for all metabolites. The average accuracies were between 86% and 120%, the RSDs for the intra- and inter-day precision were below 15% and 25% respectively. The method showed low matrix effect. Samples obtained before and after the administration of T were analyzed by both the developed UHPLC–MS/MS method and the GC–MS/MS method currently used by anti-doping laboratories. Relevant disagreements between the results obtained for 3α-glucuronide-6β-hydroxyandrosterone and 3α-glucuronide-6β-hydroxyetiocholanolone quantitation were observed. These markers seemed to be more suitable for the screening of T misuse when detected by UHPLC–MS/MS. These discrepancies were further investigated in 50 urine samples from healthy

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volunteers. The two methods gave highly correlated results for all metabolites that are currently included in the athlete's steroid profile confirming the reliability of the UHPLC–MS/MS method. However, the quantification of 3α-glucuronide-6β-hydroxyandrosterone and 3α-glucuronide-6β-hydroxyetiocholanolone, was only possible by using the UHPLC–MS/MS method since three interfering compounds were observed when performing the GC–MS/MS analysis with the most intense ion transitions. These results confirm the potential of the resistant glucuronides as biomarkers of T misuse. Additionally, they suggest that previously reported reference ranges for these metabolites should be reevaluated.

1. Introduction

The use of anabolic androgenic steroids (AAS) as doping agents has become, at least for the last 50 years, a common method among athletes in order to improve their performance [1]. Nowadays, as a response to the need of alternative biomarkers for the testosterone (T) misuse, a great body of investigation has been applied to the development of new analytical methods based on the liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) either with low or high resolution analyzers [2]. In many instances, the use of the LC–MS/MS has allowed for the direct analysis of intact phase II metabolites [3–5] and it has led to the identification of many unreported metabolites of endogenous and exogenous steroids [6–9].

Recently, the application of a UHPLC–MS/MS method revealed the presence of two glucuronides, 3α-glucuronide-6β-hydroxyandrosterone (6OH-Andros-3-Gluc) and the 3α-glucuronide-6β-hydroxyetiocholanolone (6OH-Etio-3-Gluc), which remain conjugated after enzymatic hydrolysis [10,11]. After a qualitative determination, the urinary levels of these compounds were shown to increase their concentrations around 300 fold after an oral administration of T undecanoate. Despite the fact of being resistant to enzymatic hydrolysis, basal levels of 6OH-Andros and 6OH-Etio have been reported by methods involving a hydrolysis step [12,13]. In addition, contrarily to the results obtained by the detection of the intact glucuronides, a previous study established that their concentrations remain unchanged after the administration of T [14]. In the latter cases, the results were obtained after the use of the gas chromatography-mass spectrometry (GC–MS) procedure required by World Anti-Doping Agency (WADA) for the quantification of steroids.

This GC–MS procedure, currently applied on urine samples for the screening of T misuse, includes the hydrolysis of the glucuronoconjugated metabolites of T by enzymes with β-glucuronidase activity, the derivatization of the released steroids to form the trimethylsilyl ether derivatives (keto and hydroxyl groups) and their analysis by GC–MS. The concentrations of T, its epimer epitestosterone (E), its major metabolites androsterone (Andros), etiocholanolone (Etio), 5α-androstan-3α,17β-diol (5α-Adiol) and 5β-androstan-3α,17β-diol (5β-Adiol), together with the T/E ratio calculated by this procedure compose the athlete's steroid profile as established by WADA [15]. However, although this classical GC–MS analysis is still giving satisfactory results for the quantification of steroids, several limitations have been described related with the indirect measurement of the actual amount of the steroids present in the urine [16–20].

Thus, due to the potential interest of 6OH-Andros-3-Gluc and 6OH-Etio-3-Gluc as biomarkers for T misuse, the controversial results obtained by LC and GC methodologies over their behavior after T administration should be clarified. For this reason, a UHPLC–MS/MS method was developed and validated for the direct quantification of these glucuronides. The method also included the quantification of the glucuronoconjugates of four of the main T metabolites that compose the steroid profile used in doping control analyses, namely the glucuronides of T (T-Gluc), E (E-Gluc), Andros (Andros-Gluc) and Etio (Etio-Gluc). Both the validated method and the classic GC–MS/MS method were applied to urine samples obtained before and after the oral administration of T as well as to urine samples collected from 50 healthy volunteers. The concentrations obtained by the two methods were compared in order to shed light on the divergences reported for the behavior of these metabolites.

2. Materials and methods

2.1. Chemicals

3α-glucuronide-6β-hydroxyandrosterone (6OH-Andros-3-Gluc), 3α-glucuronide-6β-hydroxyetiocholanolone (6OH-Etio-3-Gluc) and 6β-glucuronide-3α-hydroxyandrosterone (6OH-Andros-6-Gluc, used as internal standard, ISTD) were synthesized as previously described [11]. T, E, Andros, Etio, T-Gluc, E-Gluc, 5α-androstan-3α,17β-diol-3α-glucuronide, 5β-androstan-3α,17β-diol-3α-glucuronide, 5α-androstan-3α,17β-diol-17α-glucuronide, 5β-androstan-3α,17β-diol-17α-glucuronide, d_3-testosterone (d_3-T), d_3-epitestosterone (d_3-E), d_5-etiocholanolone (d_5-Etio), d_3-testosterone glucuronide (d_3-T-Gluc) and d_3-epitestosterone glucuronide (d_3-E-Gluc) were purchased from the Australian National Measurement Institute (Pymble, Australia). Andros-Gluc and Etio-Gluc were obtained from from Steraloids Inc. (Newport, RI, USA). d_4-Androsterone glucuronide (d_4-Andros-Gluc) was obtained from Orphachem (Saint-Beauzive, France). Methanol (MeOH, HPLC gradient grade), formic acid, ammonium formate (LC/MS grade), sodium bicarbonate, sodium carbonate, ammonium iodide and tert-butylmethyl ester were purchased from Merck (Darmstadt, Germany). The β-glucuronidase preparation (from *Escherichia coli* type K12) was purchased from Roche Diagnostics (Mannheim, Germany). The derivatization reagent N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Karl Bucher Chemische Fabrik GmbH (Waldstetten, Germany). 2-mercaptoethanol was purchased from Sigma–Aldrich (St Louis, MO, USA). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.2. Instrumental

2.2.1. UHPLC–MS/MS

Chromatographic separation was performed by using an Acquity UPLC instrument (Waters Associates, Milford, Massachusetts, USA). The LC system was equipped with an Acquity UPLC® BEH C18 1.7 μm 2.1 × 100 mm column (Waters Associates). The injection volume was 20 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