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A comprehensive procedure based on gas chromatography–isotope ratio mass spectrometry following high performance liquid chromatography purification for the analysis of underivatized testosterone and its analogues in human urine

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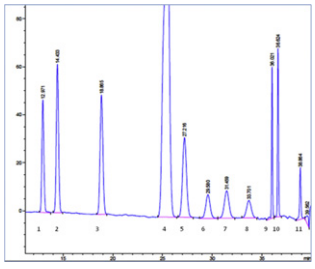
HIGHLIGHTS

- Overall approach for urine samples purification by HPLC for subsequent GC/C/IRMS analysis in doping control.
- Detection of pseudo-endogenous androgenic steroids (i.e. testosterone, androstenedione) misuse in sports.
- Routine analysis of steroids by GC/C/IRMS in sports drug testing.

GRAPHICAL ABSTRACT



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ABSTRACT

The confirmation by GC/C/IRMS of the exogenous origin of pseudo-endogenous steroids from human urine samples requires extracts of adequate purity. A strategy based on HPLC sample purification prior to the GC/C/IRMS analysis of human urinary endogenous androgens (i.e. testosterone, androsterone and/or androstenediols), is presented. A method without any additional derivatization step is proposed, allowing to simplify the urine pretreatment procedure, leading to extracts free of interferences permitting precise and accurate IRMS analysis, without the need of correcting the measured delta values for the contribution of the derivatizing agent. The HPLC extracts were adequately combined to both reduce the number of GC/C/IRMS runs and to have appropriate endogenous reference compounds (ERC; i.e. pregnanediol, 11-keto-etiocholanolone) on each GC–IRMS run. The purity of the extracts was assessed by their parallel analysis by gas chromatography coupled to mass spectrometry, with GC conditions identical to those of the GC/C/IRMS assay. The method has been validated according to ISO17025 requirements (within assay precision below 0.3‰ ^{13}C delta units and between assay precision below 0.6‰ ^{13}C delta units for most of the compounds investigated) fulfilling the World Anti-Doping Agency requirements.

1. Introduction

The use of androgenic anabolic steroids (AAS) is prohibited by the International Sport Authorities due to their potential toxicological and side effects and to defend fair play in Sports. These compounds are included in the List of Prohibited Substances and Methods edited and periodically updated by the World Antidoping

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Agency (WADA). The detection of the abuse of synthetic androgenic anabolic steroids (i.e. methyltestosterone, methandienone or stanozolol) by gas chromatography coupled to mass spectrometry (GC/MS or GC/MS/MS) has been improving in the last decades and it is not a major analytical challenge in present sports drug testing based on urine analysis [1–4]. Challenges remain, however, for the detection of the exogenous administration of synthetic androgens (the so-called "pseudo-endogenous" steroids) having the same chemical structure of the same compounds that are produced endogenously (i.e. testosterone, 5α-dihydrotestosterone or androstenedione). Methods based on the alterations of the so-called urinary endogenous steroids profiles compared to population based [5,6] or individual based [7,8] ranges have been developed. Since several factors, in addition to the exogenous administration of synthetic androgens, may alter the normal excretion of endogenous steroids [9], the need of a direct proof is necessary in order to reach a final conclusion in a short time. In the past testosterone was mainly administered in pharmaceutical preparations esterified with different fatty acids (i.e. enanthate or undecanoate) in order to modulate its pharmacokinetic properties and then if blood samples would be available the direct detection of the esters was possible during a time window that overlaps the time where the urinary steroid profiles are modified [10–12]. Since then, new synthetic androgens sold as dietary supplements have been available through internet making the interpretation of the alterations of the urinary steroid profiles more difficult. Fortunately, most of the synthetic androgens have a rather homogeneous 13C/12C ratio composition showing small differences depending on their origin (usually phytosteroids from soy) [13,14] and lower values (expressed as δ^{13}C values in ‰ units) compared to the naturally produced compounds in the human body [15–18]. Since the mid-90s the use of carbon isotope ratio mass spectrometry (IRMS) combined with gas chromatography (GC/C/IRMS) has been investigated for the confirmation of the abuse of testosterone and other synthetic endogenous steroids in sports [19,20]. The unambiguous, direct confirmation of natural endogenous steroids abuse in sports requires indeed the use of GC/C/IRMS analysis on urine samples; the drawback of the GC/C/IRMS-based analytical approach is that differences in the ^{13}C delta values are detectable in a relatively narrow window after the administration, which in turns depends also on the route of administration, producing a significant number of false-negative results. Most of these limitations can be overcome by selecting a broader number of both target steroids and endogenous reference compounds.

Attempts to define general methods for a wide-range of compounds confirmation have been made [20,21]. These methods should include the detection of the analytes of interest (the compounds and/or metabolites with modified δ^{13}C values) to be compared with endogenous reference compounds (ERC) (compounds in upper or different metabolic paths with δ^{13}C values not modified by the exogenous administration of pseudo-endogenous androgens) [19,20]. IRMS analyses impose the purity of the target compounds analysed and, consequently, the purification steps for the preparation of extracts of the adequate purity starting from a complex matrix as urine are crucial. A fundamental and practical tutorial on how to face the analyses by IRMS in doping shows the potential use of HPLC for the sample purification of the urine samples [22] for the analysis of synthetic androgens.

The aim of this work is to describe the strategy based on a simple purification step by high performance liquid chromatography (HPLC) developed and currently in use in the WADA Accredited Laboratory in Rome (Italy) for the detection of endogenous synthetic androgens to confirm their exogenous administration without the need to derivatize the extracts before their analysis by GC/C/IRMS. This strategy is used for the confirmation of atypical alterations of the androgenic steroid profiles detected by gas chromatography coupled to mass spectrometry (GC/MS).

2. Experimental

2.1. Standards and reagents

The standards of testosterone (17β-hydroxy-4-androsten-3-one, T), epitestosterone (17α-hydroxy-4-androsten-3-one, E), androsterone (3α-hydroxy-5α-androstan-17-one, A), etiocholanolone (3α-hydroxy-5β-androstan-17-one, Et), were purchased from NMIA (Pymble, Australia). 5β-androstan-3α,17β-diol (5b-Diol), 11-hydroxyandrosterone (3α,11β-dihydroxy-5α-androstan-17-one, 11OHA), 11-hydroxyetiocholanolone (3α,11β-dihydroxy-5β-androstan-17-one, 11OHE), 11-keto-etiocholanolone (3α-hydroxy-5α-androstan-11,17-dione, 11Keto), pregnanediol (5α-pregnan-3α,20α-diol, PD) and 5α-androstan-3β-ol (ISTD) were from Steraloids (Newport, RI, USA) and 5α-androstan-3α,17β-diol (5a-Diol), pregnanetriol (5α-pregnane-3α,17α,20α-triol, PT), dehydroepiandrosterone (3β-hydroxy-5-androsten -17-one, DHEA) and 17α-methyltestosterone (MT) were from Sigma–Aldrich (Milano, Italy).

All reagents and solvents (sodium hydrogen carbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate, *tert*-butylmethyl ether (TBME), acetonitrile, methanol, n-pentane, cyclohexane and isopropanol) were of analytical or HPLC grade and provided by Carlo Erba (Milano, Italy). β-Glucuronidase from *Escherichia coli* K12 was from Roche Diagnostic (Mannheim, Germany). Water was from a Milli Q water purification system (Millipore S.p.A, Milano, Italy).

CO_2 reference gas (Solgas, Monza, Italy) for isotope ratio mass spectrometer calibration was calibrated against underivatized steroids (CU/USADA34-1) with certified delta values traceable to VPDB, obtained from Prof Brenna (Cornell University Ithaca, NY).

Synthetic urine was prepared as described previously [23].

2.2. Sample preparation

Urine samples (3–6 mL) were extracted with 10 mL of *tert*-butylmethyl ether after the addition of 1 mL of phosphate buffer (0.8 M, pH 7) to separate the free from the conjugated fraction. Once the free fraction discarded, the hydrolysis was initiated by the addition of 50 μL of β-glucuronidase from *E. coli*. The hydrolysis was performed at 55 °C during 60 min. After cooling, pH was adjusted to 9–10 with carbonate buffer (20%) and extraction performed with 10 mL of *tert*-butylmethyl ether. Once the solvent separated and taken to dryness, the final residue was dissolved in 50 μL of a mixture water/methanol (50/50) containing methyltestosterone (100 μg mL^{-1}) for subsequent HPLC purification of the extract.

2.3. HPLC sample purification

Sample purification was performed using an ACE 5 C18 column (CPS Analitica, Milano, Italy) (25 cm, 4.6 mm, 5 μm) and an ACE 5 C18 guard cartridge at 38 °C. Separation was programmed with a mobile phase composed with water (solvent A) and acetonitrile (solvent B). For compounds separation, an isocratic programme was set up at 38% B for 31 min then increasing to 55% B in 0.01 min, then to 65% B in 4 min and kept at 65% B for additional 4 min. The column was flushed for 5.5 min at 100% B and finally re-equilibrated at 38% B for 5 min for a total run time of 47 min. The flow rate was set at 1 mL min^{-1}.

All separations are performed in an Agilent 1100 Series liquid chromatograph (Agilent Technologies SpA, Cernusco sul Naviglio, MI, Italy) and the selected fractions collected in a Agilent 1100