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Development of a GC/C/IRMS method – Confirmation of a novel steroid profiling approach in doping control

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ABSTRACT

In doping control, an athlete can only be convicted with the misuse with endogenous steroids like testosterone (T), if abnormal values of steroid metabolites and steroid ratios are observed and if the subsequent analysis with isotope ratios mass spectrometry (IRMS) confirms the presence of exogenously administered androgens. In this work, we compare the results of a novel steroid profiling approach with the performance an in-house developed IRMS method. The developed IRMS has the advantage over other methods to be relatively short in time and with target compounds androsterone, etiocholanolone, 5β-androstane 3α,17β-diol and 5α-androstane 3α,17β-diol. Pregnanediol was used as an endogenous reference compound (ERC).

Reference limits for the IRMS values were established and applied as decision limits for the evaluation of excretion urine from administration with oral T, T-gel, dihydrotestosterone (DHT) – gel and dehydroepiandrosterone (DHEA). Results indicated the importance of both androstanediols as important IRMS markers where relative values compared to an ERC ($\Delta\delta^{13}C$) yielded better detection accuracy than absolute $\delta^{13}C$-values. The detection times of all administered endogenous steroids were evaluated using the proposed thresholds.

The results of traditional steroid profiling and a new approach based upon minor steroid metabolites monitoring introduced in a longitudinal framework were evaluated with IRMS. With traditional steroid profiling methods, 95% of the atypical samples could be confirmed whereas an additional 74% of IRMS confirmed was provided by a new biomarkers strategy.

These results prove that the other steroid profiling strategies can improve the efficiency in detection of misuse with endogenous steroids.

1. Introduction

It is well known that athletes may abuse anabolic androgenic steroids with the intent of enhancing athletic performance, through increased muscle mass and faster recovery from injury or intense training [1]. As a consequence, these substances are prohibited by international sporting organisations and the World Anti-Doping Agency [2]. In addition, the disruption of the natural biochemical balance within the human body imposes serious health risks, making the abuse of synthetic steroids illegal in many countries [3,4]. Testosterone (T) is probably the most widely misused steroid among athletes [5] and is easily accessible. Moreover, detection of misuse with endogenous steroid is a challenging task as these substances are usually administered in small doses and are structurally identical to those produced by the body.

Advanced and innovative analytical techniques centred on gas chromatography-mass spectroscopy (GC–MS) have been very successful at identifying and quantifying testosterone and its main metabolites in urine samples. Therefore, GC–MS is the method of choice to determine the misuse of endogenous steroids among athletes for doping control analysis. Investigation of the GC–MS urinary steroid profile, which consists at least of T and the metabolites androsterone (A; 3α-hydroxy-5α-androstane-17-one), etiocholanolone (Et; 3α-hydroxy-5β-androstane-17-one), 5β-androstane-3α,17β-diol (βαβ-diol), 5α-androstane-3α,17β-diol (ααβ-diol) and epitestosterone (E; 17α-hydroxyandrost-4-ene-3-one), allows analysts to identify athlete samples with suspicious values [6–8]. As several endogenously produced steroids like dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione (Adion) and androstenediol became available as food supplements or pharmaceutical formulations, dishonest athletes found an easy way to purchase these androgens of which produc-

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ers claimed they possess ergogenic properties. Administration studies performed with these endogenous steroids demonstrated the pertinence of additional markers to be monitored in steroid profile screening in sport drug testing laboratories [9,10]. To improve the detection specificity, steroid profiling methods were presented that included minor steroid metabolites [11].

Nevertheless, GC–MS steroid profiling cannot differentiate between endogenous steroids and their synthetic analogs. As a consequence, doping control laboratories, accredited by the World Anti-Doping Agency (WADA), have been confronted with the task to develop analytical methods and establish criteria that unequivocally distinguish endogenous steroids from their synthetic copies.

It has been known for some time that gas chromatography-combustion-isotope ratio mass spectrometry (GC–C–IRMS) is capable of meeting this challenge, as well as the strict analytical demands in doping control [12–14]. Synthetic testosterone derivates, generally derived from plant sterols stigmasterol and sitosterol obtained from soybean (Glycine max), have a significantly different carbon isotope composition compared to endogenously produced steroids [15–17]. As a consequence, determination of misuse of synthetic analogs of natural steroids can be established by measuring the carbon isotopic composition of testosterone and/or its metabolites. In practice, the measured $^{13}C/^{12}C$ values of the target analytes, which suffer from large inter-individual biological variations, are compared with other urinary steroids produced by different metabolic pathways. These so-called endogenous reference compounds (ERC's) are not affected by the intake of synthetic anabolic androgenic steroids and reflect the basal carbon isotope ratios (CIR) of a subject [18]. In doping control analysis, typical ERC's include 5β-pregnanediol (PD) [19,20], 11-oxo-etiocholanolone [21] and 11β-hydroxyandrosterone [22]. The main target compounds (TC's) are A, Et, βαβ-diol and ααβ-diol [23]. Unfortunately GC–C–IRMS remains a very laborious and expensive technique which also demands substantial amounts of urine to meet the sensitivity requirements of the WADA. Due to these facts, GC–C–IRMS remains a confirmation procedure and is only applied to suspicious, i.e. atypical, urine samples ([23]).

In doping control, recent advances involving longitudinal programs and statistical discrimination algorithms have proven to be a substantial leap forward in detecting suspicious steroid profiles. However, the true outcome of such atypical steroid profile can only be confirmed by stable isotope measurements. In this work, we present a relatively fast GC/C/IRMS method employing a single SPE and single HPLC fraction collection step. The IRMS method was used to corroborate the findings of these novel screening strategies with the WADA IRMS standards. Reference ranges of the TC $^{13}C/^{12}C$ values were calculated and applied for optimal detection performance in a probabilistic framework.

2. Experimental

2.1. Reagents and chemicals

$NaH_2PO_4.H_2O$, $Na_2HPO_4.2H_2O$, Na_2SO_4 and K_2CO_3 were purchased from Merck (Darmstadt, Germany). Ethyl acetate, cyclohexane, LC-MS grade methanol and $NaHCO_3$ were obtained from Fisher Scientific (Leicestershire, UK) and diethyl ether, LC–MS grade acetonitrile and LC–MS grade water from Biosolve (Valkenswaard, The Netherlands). β-Glucuronidase (E. coli) was purchased from Roche Diagnostics (Mannheim, Germany); helium, carbon dioxide and oxygen from Air Liquide (Bornem, Begium); acetic anhydride, pyridine, etiocholanolone (Et), androsterone (A), androsterone acetate (A-Ac), 5β-androstan-3α,17β-diol (βαβ-diol), 5α-androstan-3α,17β-diol (ααβ-diol), 5β-pregnan-3α,20α-diol (PD), 5β-pregnan-3α,20α-diol diacetate (PD-Ac_2), 17β-Trenbolone (β-Tren) and 5-androstene-3β,17α-diol (5-en) from Sigma Aldrich (St. Louis, MO, USA) and the n-alkane calibration mix (C_{17}-C_{25}) was provided by the Biogeochemical Laboratories of the Indiana University (Bloomington, IN). 17α-methyl testosterone (MeT) was obtained from Organon (Oss, The Netherlands) and etiocholanolone acetate (Et-Ac), 5β-androstan-3α,17β-diol diacetate (βαβ-Ac_2), 17β-Trenbolone acetate (β-Tren-Ac), 5α-androstan-3β-ol (5α-ol) and 5α-androstan-3β-ol acetate (5α-ol-Ac) from Steraloids (Newport, USA). All steroid standards contained less than 1% impurities. All standard solutions were made in methanol, except 5α-ol-Ac which was dissolved in cyclohexane and stored at 4 °C.

2.2. Steroid profiling and concentration estimation

In order to estimate the urinary steroid concentrations, aliquots of 5 ml were analysed for their free and glucuronide fractions on GC/MS according to a protocol published earlier [11]. The urinary steroid concentration allowed us to estimate the necessary sample volume for optimal IRMS measurement. Using the measured βαβ-diol concentration ($C_{\beta\alpha\beta\text{-diol}}$) in the urine samples, a good estimate for the required sample volume (V_{IRMS}) for IRMS was obtained using following relation:

$$V_{IRMS} = \frac{1250}{C_{\beta\alpha\beta-\text{diol}}}$$

2.3. Pre-analytical steps

Additional to the compounds screened during the steroid profiling step, PD was also targeted for IRMS analysis as the ERC of choice.

A system blank, QC negative and QC positive were aliquoted with each batch of urine samples. 50 μL of MeT (100 μg/mL) internal standard (IS) was added to the urine aliquot (2–25 mL), followed by centrifugation. Before the urine was loaded on the solid phase extraction (SPE) cartridge (Bond Elut C18, 500 mg, 3 mL, Agilent technologies), two conditioning steps (5 mL CH_3OH, 5 mL H_2O) were carried out. Two washing steps were conducted (5 mL H_2O, 5 mL 10% CH_3OH/H_2O) and the compounds of interest were eluted (5 mL CH_3OH). The methanolic extract was evaporated to dryness under nitrogen at 60 °C and reconstituted in 1 mL of a 0.1 M pH 7 phosphate buffer. 50 μL of β-glucuronidase enzyme was added and hydrolysis took place in an oven at 56 °C for 60 min. The sample was cooled to room temperature before 1 mL $NaHCO_3/K_2CO_3$ buffer (pH 9.5) and 5 mL diethyl ether were added. LLE was performed for 20 min, followed by centrifugation. Afterwards the organic phase was transferred to a new tube, dried by adding anhydrous Na_2SO_4 and again transferred to a new tube which contained 50 μL β-Tren (100 μg/mL) IS. An additional IS sample was prepared by transferring 50 μL of A, Et, βαβ-diol, ααβ-diol, PD, MeT and β-Tren (100 μg/mL) IS to a new tube. This sample provided an extra verification that the retention times on which the fraction collection was based on, were indeed correct. Consequently, the organic phase was evaporated under nitrogen at 40 °C. Acetylation took place by adding 50 μL of acetic anhydride and 50 μL of pyridine (60 min at 80 °C). The acetylation reagents were evaporated under nitrogen at 60 °C and the residue was reconstituted in 120 μL of 75/25 CH_3OH/H_2O and filtered with a micro-centrifuge before being transferred to an LC vial.

2.4. HPLC fraction collection

To further purify the sample, a semi-preparative HPLC fraction collection was performed on a Thermo Scientific Surveyor (Bre-