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Analytical approach for the determination of steroid profile of humans by gas chromatography isotope ratio mass spectrometry aimed at distinguishing between endogenous and exogenous steroids

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ABSTRACT

The contamination of commonly used supplements by unknown steroids as well as their metabolites (parent compounds) become a challenge for the analytical laboratories. Although the determination of steroids profile is not trivial because of the complex matrix and low concentration of single compound, one of the most difficult current problem is to distinguish, during analytical procedure, endogenous androgens such as testosterone, dehydrotestosterone or dehydroepiandrosterone from their synthetic equivalents.

The aim of this work was to develop and validate an analytical procedure for determination of the steroid profile in human urine by gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS) toward distinguishing between endogenous and exogenous steroids. Beside the optimization of the experimental parameters for gas chromatography separation and mass spectrometry, attention was focused on urine sample preparation. Using an optimized sample preparation protocol it was possible to achieve better chromatographic resolutions and better sensitivity enabling the determination of 5 steroids, androsterone, etiocholanolone, testosterone, 5-androstandiol, 11-hydroxyandrdostane, pregnandiol, with the expanded uncertainty ($k = 2$) below 1‰. This enable to evaluate the significant shift of the $\delta^{13}C/^{12}C$ [‰] values for each of examined steroids (excluding ERC). The analytical protocol described in this work was successfully used for the confirmation of positive founding urine by evaluation T/E ratio after GC/C/IRMS analysis.

1. Introduction

Several anabolic androgenic steroids (AAS) belong to the group of synthetic compounds and their chemical structure is similar to that of natural anabolic steroid testosterone. Anabolic steroids become popular after isolation and elucidation of the chemical structure of the mother compound testosterone, the principal male sexual hormone, in 1930s [1]. Later on in 1935, Ernst Lacqueur and his coworkers isolated a testosterone as a crystal pure substance. Testosterone, the predominant circular androgen, is not only an active hormone but also a prohormone for more active androgen, 5α-reduced steroid dihydrotestosterone (DHT) [2,3].

The most frequently used method for the determination of anabolic agents is gas chromatography with mass spectrometry (GC/MS), although in certain cases it is possible to obtain better limit of quantification (less than 2 ng/mL) using liquid chromatography tandem mass spectrometry (LC–MS/MS) [4]. In both cases, the primary requirements is to achieve sufficient resolution of chromatographic separation of steroids. Moreover the adequate sensitivity of the detector enabling the determination of ^{13}C to ^{12}C carbon isotopes ratio (δ^{13}‰) should be possible. As the value of δ^{13} enables distinction between endogenous and exogenous steroids, GC/C/IRMS become a method of choice. It was found that δ^{13} around (−22 to −24)‰ is typical for endogenous steroids being a result of natural biosynthesis and (−27 to −29)‰ for exogenous synthetic steroids. However, before the final conclusions it is essential to be aware that exact values depends on a diet and a country of origin of a sportsmen [5].

Abbreviations: AAS, anabolic–androgenic steroids; GC/C/IRMS, gas chromatography combustion isotope ratio mass spectrometry; T/E, testosterone to epitestosterone ratio.

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Because of the low concentration of AAS as well as a complex matrix of the human urine, before the GC/C/IRMS is applied, extensive sample preparation is required. The common scenario described in the literature consist of the preconcentration of steroids followed by their separation via a multi-step extraction, optionally with the use of preparatory HPLC. The most essential steps of the sample preparation procedure are: (i) preliminary extraction of analytes by solid phase extraction (SPE); (ii) addition of buffer to obtain respective pH; (iii) enzymatic hydrolysis and; (iv) final extraction by liquid–liquid extraction (LLE). Thus, the optimization of the conditions used during the sample preparation requires careful selection of solid phase for SPE, buffer used for the stabilization of pH, enzyme used for hydrolysis, organic solvent used for LLE as well as internal standards for MS detection.

According to the literature, the SPE was performed on octadecyl sorbent (C18), followed by the use of methanol [6–12], isopropanol [13], ethyl acetate or acetonitryl [14–17] for elution. Enzymatic hydrolysis was performed with β-glucuronidase—*Escherichia coli* [6–8,12,13] or *Helix pomatia* [16]. For the liquid extraction, diethyl ether [14,18] or methyl *tert*-buthyl ether [19–21] were used. Optionally, preparatory HPLC was used when extensive cleaning of the urine samples is needed. If so octadecyl (RP-C18), 125 × 4.0 mm [5,22,23] or 250 × 4.6 mm [7,24–26] with 5 μm were applied. In some work derivatization with bismethylenedioxypentafluoropropionate (BMD-PFP) [14], acetic anhydrite in 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol [13,15,17], were used.

Although the number of publications dealing with sample preparation procedure are available, to the best of our knowledge no systematic investigations on its detail optimization were considered. The aim of this work was to validate via systematic investigations of the human urine preparation, toward establish the standardized procedure for sample pretreatment, followed by the determination of steroids profile by GC/C/IRMS.

2. Materials and methods

2.1. Equipments

Isotope ratio mass spectrometer (IRMS) Delta V Advantage (Thermo Fisher Scientific GmbH Bremen, Germany) coupled with gas chromatograph Trace GC Ultra equipped with GC Combustion III (Thermo Fisher Scientific GmbH Bremen, Germany) was used. The chromatographic separation of steroids were performed on the DB-5 column 30 × 0.25 mm, 0.25 μm (Agilent Technologies Willington, USA). The following experimental parameters were used: helium was used as a carrier gas at 1.8 mL/min at 140 °C. Splitless injection of 2 μL of the sample solution at 270 °C was used.

The temperature was increased during 2 min from 140 °C to 300 °C with the following gradients: (i) 60 °C/min till 200 °C; (ii) 10 °C/min, till 300 °C/hold 2 min.

The combustion was performed in oxidizing conditions at 850 °C, followed by reduction conditions at 25 °C. For the electron ionization (EI) 70 eV was used, as the temperature of ion source was 260 °C. Ions monitoring (*m/z*): 44, 45 and 46.

As GC/MS system gas chromatography (7890A Agilent Technologies) with mass spectrometry (5795 C Agilent Technologies) was used. As column HP1 17 m × 0.2 mm × 0.11 μm; Agilent Technologies was installed. The mobile phase was helium at a constant flow-rate of 1 mL/min. 2 μL of the sample was injected in the split mode (1:10). The initial temperature of 180 °C was held for 0.3 min and subsequently increased to 231 °C at a constant rate of 3 °C/min. Next, the temperature was further increased to 310 °C (30 °C/min) and held for 2 min. The temperature of transfer line and ions source were 310 °C and 230 °C, respectively. The selected ion-monitoring (SIM) mode was used.

2.2. Reagents

5β-androstane-3α, 17β-diol(5β-androstendiol), 5α-androstane-3α, 17β-diol(5α-androstendiol), epitestosterone and etiocholanolone were from Steraloids (Newport, USA); pregnandiol and 11-hydroxyandrosterone (11-OHAndro) were from Merck (Darmstadt, Germany); androsterone was from Sigma–Aldrich (Poznan, Poland); 11-ketoetiocholanolone (11-ketoEtio), 11-hydroxyetiocholanolone (11-OHEtio) and dehydroepiandrosterone were from Sigma–Aldrich (Poznań, Poland); testosterone was from Polfa (Warsaw, Poland). All standard solutions contains 1 mg/mL of respective compound in methanol. The solutions of steroids were well seal and protect by parafilm, then was kept at 4 °C.

Methanol, ethanol, water, diethyleter, methyl *tert*-buthyl ether, *n*-penthane, *n*-hexane; all of purity required by HPLC J. T. Baker, S. Witko (Łódź, Poland).

Fosforic Buffer of 0.2 mol/L (Na_2HPO_4/NaH_2PO_4, cz.d.a., pH = 7); POCH SA (Gliwice, Poland).

IRMS buffer (20% $K_2CO_3/KHCO_3$, cz.d.a.); POCH SA (Gliwice, Poland).

β-glucuronidase *E. coli* and *H. pomatia* from Roche Diagnostics GmbH (Mannheim, Germany).

Gases: He from Linde (Warsaw, Poland); N_2 from BOC Gazy Siewierz (Siewierz, Poland); CO_2 and O_2 from Messer (Chorzow, Poland).

Following sorbents were used within this work:

C-18 from J. T. Baker (12 μm, 60A, Octadecyl, Strongly Non Polar, 500 mg/6 mL).

Strata C-18-E; Phenomenex Torrance, USA (55 μm, 70A, 500 mg/6 mL).

Strata-X-C; Phenomenex Torrance, USA (33 μm, Polymeric Strong Cation, 200 mg/6 mL).

Strata-X; Phenomenex Torrance, USA (33 μm, Polymeric Reversed Phase, 200 mg/6 mL).

2.3. Treatment of urine samples

Two control samples containing 25 μg/mL of each steroids in ethanol were prepared from the standard solutions: sample labeled as SM1 contains synthetic androsterone, etiocholanolone, testosterone and pregnandiol; sample labeled as SM2 contains 5α- and 5β-androstendiol, epitestosterone, 11-ketoetiocholanolone, 11-hydroxyandrosterone and 11-hydroxyetiocholanolone. Both control samples were used in order to establish retention time for each compound as well as its $\delta^{13}C/^{12}C$ [‰] (Table 1). Note that the compounds marked with (*) are the reference compounds used as a standard for the calculation the shift of the $\delta^{13}C/^{12}C$ [‰].

The humane urine free of exogenous synthetic steroids (sample label as BU—blank urine from 26 old male voluntaries), without (BU) or with a standards additions (BU + SM1/BU + SM2) was used for the optimization of the analytical procedure.

The number of humane urines from athletes (label as Urines 00X), suspected of having the elevated level of exogenous synthetic steroids, were used for the examination of the validity of the either procedure.

All samples were undergoes the follows sample pre-treatment: (i) solid phase extraction (SPE); (ii) preliminary liquid–liquid extraction; (iii) enzymatic hydrolysis; (iv) final liquid–liquid extraction.

2.4. Validation of the analytical procedure

The fit for purpose validation process was involved for the determination of the carbon isotopic ratio toward the calculation of the $\delta^{13}C/^{12}C$ [‰]. The chromatographic resolution and the uncertainty of the determination of the carbon isotopic ratio used