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Gas chromatographic quadrupole time-of-flight full scan high resolution mass spectrometric screening of human urine in antidoping analysis

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ABSTRACT

This paper presents the development and validation of a high-resolution full scan (FS) electron impact ionization (EI) gas chromatography coupled to quadrupole Time-of-Flight mass spectrometry (GC/QTOF) platform for screening anabolic androgenic steroids (AAS) in human urine samples. The World Antidoping Agency (WADA) enlists AAS as prohibited doping agents in sports, and our method has been developed to comply with the qualitative specifications of WADA to be applied for the detection of sports antidoping prohibited substances, mainly for AAS. The method also comprises of the quantitative analysis of the WADA's Athlete Biological Passport (ABP) endogenous steroidal parameters. The applied preparation of urine samples includes enzymatic hydrolysis for the cleavage of the Phase II glucuronide conjugates, generic liquid–liquid extraction and trimethylsilyl (TMS) derivatization steps. Tandem mass spectrometry (MS/MS) acquisition was applied on few selected ions to enhance the specificity and sensitivity of GC/TOF signal of few compounds. The full scan high resolution acquisition of analytical signal, for known and unknown TMS derivatives of AAS provides the antidoping system with a new analytical tool for the detection designer drugs and novel metabolites, which prolongs the AAS detection, after electronic data files' reprocessing. The current method is complementary to the respective liquid chromatography coupled to mass spectrometry (LC/MS) methodology widely used to detect prohibited molecules in sport, which cannot be efficiently ionized with atmospheric pressure ionization interface.

1. Introduction

Anabolic Androgenic Steroids (AAS) are the most frequently used class of prohibited substances by athletes [1,2] to boost their performance in sport activities. The detection of AAS in athletes' urine is a challenge for the doping control laboratories because of a) the low concentrations of the precursors and their metabolites, b) the low Minimum Required Performance Limits (MRPL) requested by the World Anti-Doping Agency (WADA) [3], c) the availability of designer steroids, which have similar activity and same or different chemical composition (formula) with known (not necessarily endogenous) steroids but different structures [4,5], d) the continuous discovery of new long-term metabolites of AAS that extend the retrospectivity of the consumption considerably (however the incorporation of these new metabolites in screening procedure is really helpful for the monitor of steroids abuse and has led in the past to numerous adverse analytical findings) [6–9], and e) the rumored use of "micro dosing", where athletes are doped with small doses which provide concentration in body fluids sampled for anti doping below the detection limit.

The doping control laboratories implement different analytical techniques in order to be able to detect a large variety of classes of prohibited substances. Mass spectrometry is the method of choice for the detection of the small molecules present in prohibited list of substances [1] combined either with gas chromatography (GC/MS) or with liquid chromatography (LC/MS). Due to their limited ionization efficiency, AASs are screened by GC/MS [10,11] and LC/MS are used for AASs, which can be efficiently ionized and hence selectively detected [12]. Only few free AASs are efficiently ionized, but there are numerous recent examples for the detection of intact Phase II metabolites of AASs by LC/MS analysis [13]. Regarding the LC/MS screening, WADA

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accredited laboratories use either LC triple quadrupole MS (LC/QQQ) [14,16] or LC High Resolution MS (LC/HRMS) − orbitrap or TOF − mass analyzers [15,17,18]. During the Olympic Games of London 2012 and Rio 2016, LC/HRMS orbitrap technology was used [18]. On the contrary, regarding the GC/MS screening, merely low resolution GC triple quadrupole technology (GC/QQQ) is used [10,11], while GC/HRMS has been proposed for special purposes as for the detection of Xenon [19].To the best of our knowledge, there is only one article regarding the use of full scan (FS) GC/HRMS technology as screening tool in the doping control field [20]. The main advantages of FS HRMS compared to triple quadrupole technology are the significantly reduced background noise originating from the urine matrix, the high resolving power and mass accuracy used as additional identification information and the capability to perform retesting of the samples by simply reprocessing the stored data files, whenever there is a special request for this such as when a new doping substance or its metabolite is discovered. The last feature (retesting) is gaining importance in sports drug testing, due to the impressive results that came out after the retesting of samples from the Beijing 2008 and the London 2012 Olympic Games some weeks before the Rio 2016 Olympic Games took place. While the percentage of positive cases coming from the original analysis of samples during the Games was far less than 1% (0.13% in Beijing 2008 [21], 0.16% in London 2012 [22]), the reanalysis of samples in 2016 of 1243 samples from Beijing 2008 and London 2012 Games that was reported previously negative, lead to additional identification of 98 positive cases, constituting an astonishing percentage of 8% [23].

In addition to screening for the exogenous compounds, GC/MS screening is used for the quantification of markers of the urinary WADA Athlete Biological Passport (ABP) Steroid Profile (SP) [24]. Currently, the SP consists of Testosterone (T), Epitestosterone (E), Androsterone (A), Etiocholanolone (Etio) 5α-androstan-3α,17β-diol (5αadiol), 5β-androstan-3α,17β-diol (5βadiol), as well as the ratios T/E, A/Etio, 5αadiol/5βadiol, A/T, 5αadiol/E. The analytical method used should be fit-for-purpose and allow covering the dynamic concentration range of listed compounds determined in both males and females. This means that the method should be able to quantify concentrations ranging from 2 ng/mL to more than 10 μg/mL in a single aliquot. WADA has set specific and strict requirements for the methods used for the quantification of the SP markers [24]. The implementation of GC/HRMS for quantification of the compounds listed in ABP-SP presents a challenge in terms of the dynamic range of currently available instruments.

In this paper, we described the use of high-resolution full scan gas chromatographic quadrupole Time-of-Flight mass spectrometry (GC/QTOF) to be used as screening platform for doping control purpose using FS and HRMS data. The method is validated for 73 analytes − mainly AAS but other categories of prohibited substances as well − at concentrations levels at or below the WADA MRPL [3]. Furthermore, this method is used for the quantification of the parameters of the SP to be included in the ABP [24]. To assess the performance of FS HRMS GC/QTOF approach, we present a comparison between GC/QQQ and GC/QTOF profiling of SP measured in the same sample set. The prospect and the feasibility of the implementation of FS obtained with HRMS as a routine screening method is discussed with aim to substitute the triple quadrupole method for doping control purpose.

2. Experimental

2.1. Material and methods

2.1.1. Reagents

Sodium hydrogen carbonate and diethyl ether were supplied by Merck (Darmstadt, Germany). Methanol (HPLC grade), 2-Propanethiol, di-potassium hydrogen phosphate trihydrate ($K_2HPO_4{\cdot}3H_2O$), potassium dihydrogen phosphate (KH_2PO_4), ammonium iodide (NH_4I), sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) were supplied by Sigma Aldrich (Darmstadt, Germany). β-Glucuronidase from *Escherichia Coli* (E.coli) was supplied by Roche (Mannheim, Germany). MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) was supplied by Chemische Fabrik Karl Bucher (Waldstetten, Germany). Perfluorotributylamine (PFTBA) from Agilent.

2.1.2. Reference materials

The following internal standards (ISTD) were purchased from LGC (Wesel, Germany): etiocholanolone-D5 (d5 Etio), androsterone glucuronide-D4 (d4A Glu), testosterone-D3 (d3T), epitestosterone-D3 (d3E), 5β-androstane-3α-17β-diol-D5 (D5-5βAdiol). The remaining reference materials of the study were purchased from LGC (Wesel, Germany), TRC (Toronto, Canada), Sigma Aldrich (Darmstadt, Germany), Steraloids (Newport, USA), and Cerilliant (Round Rock, USA). Stock standard solutions of the analytes were individually prepared in methanol. For validation purposes, working standard solution containing the analytes was prepared in methanol by subsequent dilutions of the stock solutions. All solutions were stored at −20 °C in amber vials. The steroid profile analytes were included in a different working solution.

Urine samples from excretion study of dehydrochloromethyltestosterone (oral turinabol), Desoxymethyltestosterone (Madol), Oxymetholone, Mathandienone, Oxandrolone were donated by the Doping Control Laboratory of Athens, Greece or provided by the World Association of Antidoping Scientists (WAADS).

2.1.3. Sample preparation

Two and a half (2.5) mL of urine aliquot is hydrolyzed by 50 μL of beta-glucuronidase enzyme from *E. Coli* and incubated for 90 min in 50 °C after the addition of 25 μL of ISTD mixture (d3T, d3E, d4A Glu, d5 Etio, d5-5βAdiol) and 1 mL pH 7 phosphate buffer that was prepared by adding 169.8 g of $K_2HPO_4{\cdot}3H_2O$ and 54 g KH_2PO_4 in 1 L of water. After hydrolysis, the urine is buffered by $NaHCO_3$: Na_2CO_3 (10:1) and extracted at pH 9–10 by 5 mL diethyl ether. The sample is centrifuged at 3000 rpm for 12 min and the organic phase is separated from the aqueous phase in frozen conditions at −80 °C and evaporated under nitrogen flow at 50 °C. The residue was TMS derivatized by adding 50 μL of derivatization reagent MSTFA/NH_4I/2-Propanthiol (1000:4:8) and was incubated in 100 °C for 60 min.

2.1.4. Instrumentation

2.1.4.1. GC/QTOF. The GC/MS system used in the current study is an Agilent GC 7890 coupled with an Agilent 7200 QTOF MS (G3850-64101) equipped with 5% Phenyl polysilphenylene-siloxane capillary column (30 m length, 0.25 mm ID, 0.1 μm film thickness, SGE BP X5) and back flush system. The quadrupole device prior the TOF MS analyzer provides the capability of applying MS/MS experiments. Helium was used as carrier gas with a constant flow set at 1.1 mL/min. Two microliters were injected in split mode of 20:1. The injection port and the interface temperatures were set at 280 °C. Initial oven temperature was 160 °C, ramped at 10 °C/min to 200 °C, then ramped at 2 °C/min to 220 °C, ramped at 6 °C/min to 292 °C, 50 °C/min up to 310 °C and held for 3 min, total run time 29.36 min. Two (2) GHz extended dynamic range (EDR) acquisition mode was used for TOF data acquisition. The acquisition rate was 5 spectra per sec, 200 msec per spectrum, number of transients per spectrum was 2718. The used GC/MS has the capacity of acquiring MS data in high-resolution FS mode with a mass accuracy < 5 ppm mass error in EI mode depending on the concentration of the analytes. The MS range (80–670 *m/z*) is capable of covering MS acquisition of all small molecules analyzed by the GC/QTOF. To correct for an eventual shift in *m/z*, a mass calibration procedure was introduced in the analysis sequence after every three aliquot injections. The instrument calibrator was Perfluorotributylamine (PFTBA, Agilent).