Steroids 78 (2013) 1226–1232

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Speeding up the screening of steroids in urine: Development of a user-friendly library

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ARTICLE INFO

Article history:
Received 3 June 2013
Received in revised form 14 August 2013
Accepted 23 August 2013
Available online 11 September 2013

Keywords:
Androgenic anabolic steroids
MALDI-TOF–MS/MS
MLibrary software
Bioinformatics
Anti-doping

ABSTRACT

This work presents a novel database search engine – MLibrary – designed to assist the user in the detection and identification of androgenic anabolic steroids (AAS) and its metabolites by matrix assisted laser desorption/ionization (MALDI) and mass spectrometry-based strategies. The detection of the AAS in the samples was accomplished by searching (*i*) the mass spectrometric (MS) spectra against the library developed to identify possible positives and (*ii*) by comparison of the tandem mass spectrometric (MS/MS) spectra produced after fragmentation of the possible positives with a complete set of spectra that have previously been assigned to the software. The urinary screening for anabolic agents plays a major role in anti-doping laboratories as they represent the most abused drug class in sports. With the help of the MLibrary software application, the use of MALDI techniques for doping control is simplified and the time for evaluation and interpretation of the results is reduced. To do so, the search engine takes as input several MALDI-TOF–MS and MALDI-TOF–MS/MS spectra. It aids the researcher in an automatic mode by identifying possible positives in a single MS analysis and then confirming their presence in tandem MS analysis by comparing the experimental tandem mass spectrometric data with the database. Furthermore, the search engine can, potentially, be further expanded to other compounds in addition to AASs. The applicability of the MLibrary tool is shown through the analysis of spiked urine samples.

1. Introduction

The use of androgenic anabolic steroids (AAS) and hormones to enhance athletic performance has important health and social implications. Their use was first introduced in sports as agents supporting the athlete recuperation after extreme stress and fatigue, but rapidly became the main agents used in doping abuse [1].

Nowadays, this class of drugs is a major group included in the prohibited list of the world anti-doping agency (WADA) as well as of major sports authorities [2–5]. In the WADA statistic report for 2011, the AAS represented 59.4% of all adverse analytical findings reported by WADA accredited laboratories [6]. Although this data may not reflect the real doping abuse statistical status, because of the well-known problems in the detectability of clandestinely designed AAS, micro dosages of endogenous AAS and "modern" doping agents (e.g., peptide hormones) [1,7–9].

The use of AAS to increase muscle mass and strength is not a behaviour strictly related to elite athletes, as their use is increasing amongst amateur athletes as well as outside sports as an expression of an improved life style [10,11]. The illicit AAS use is an increasing trend in western societies and the emergent AAS dependence is a matter of growing public health concern [12].

Quickly following the development of mass spectrometry (MS) detectors, its use coupled to gas chromatography (GC) has become the standard technique for AAS control. Currently, most methods for routine detection of these compounds and their metabolites, comprising both screening and confirmatory analysis, are still based in GC–MS techniques [13–17]. More recently, due to the increasing complexity of doping analyses and in order to enhance the detection of this group, liquid chromatography coupled with MS/MS is gaining ground within anti-doping laboratories [18–20]. In particular for the detection of thermo-labile and polar steroids, such as Trenbolone. It avoids the derivatisation step required by GC–MS and provides good sensitivity for the determination of these compounds [21,22]. Moreover, the low throughput provided by LC–MS techniques are to some extent being surpassed by the introduction of uHPLC systems coupled with LC-columns containing solid core particles that allows high speed and high efficiency

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0039-128X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.steroids.2013.08.014

separations. Unfortunately, for the majority of the AAS, due to their poor ionisation efficiencies, their determination by LC–MS may lead to losses in sensitivity.

The combination of these two factors, the long separation times of gas chromatographic techniques and the increasing workloads within anti-doping laboratories, expose an urgent need for an analytical technique allowing simplicity, speed and high throughput for the screening of the huge number of banned compounds, particularly the AAS.

Recently, the use of matrix-assisted laser desorption/ionization (MALDI) for the analysis of small molecules, has grown as a potential technique, which is reflected by the increasing number of studies reported in literature [23–27]. Moreover, it appears extremely promising for high-throughput, which is a major demand for future anti-doping methods.

In light of the latest technological improvements of this analytical technique we have recently study the applicability of a wide variety of commercial MALDI matrices for the rapid screening of AAS [28]. The matrix 2-(4-hydroxyphenylazo)-benzoic acid (HABA) was found to be the most robust for the analysis of anabolic steroids after a derivatisation step with the reagent Girard T hydrazine. The Girard T hydrazone derivative produced after derivatisation is a quaternary ammonium ion that originates a strong $[M]^+$ ion signal in the MALDI mass spectrum, as a result it increases the intensity of the steroid signal. In the aforementioned work it was demonstrated that positive identification of the characteristic peaks for all the compounds studied is possible for a sample concentration of 10 ng/mL in the MALDI sample plate. The sensitivity achieved with the HABA matrix after derivatisation was similar to that achieved by GC/MS – around 4–10 ng/mL in the single ion monitoring mode.

In the present work it is presented a step forward in simplifying AAS control through the use of and easy sample treatment and friendly software. The software is freely source code available, and it can be run as a multiple platform. As a proof-of-concept, the rapid screening of AAS in urine is reported using a sample treatment previously published by our team [28].

2. Material and methods

2.1. Chemicals

Standards of 17-α-methyltestosterone and nandrolone were purchased from Riedel-de Haën (Seelze, Germany). The standards 17α-trenbolone, 2α-methyl-5β-androstane-3α-ol-17-one, mesterolone, methandienone, calusterone, fluoxymesterone, ethisterone and mibolerone were kindly provided by the Portuguese National Anti-doping Laboratory and the Italian National Anti-doping Laboratory. A solution of β-glucuronidase from *Escherichia coli K12* with a specific activity approximately of 140 U/mg at 37 °C and pH 7 with nitrophenyl-β-D-glucuronidase as substrate (1 mL contains at least 140 U) was purchased from Roche Diagnostic (Mannheim, Germany). Sodium hydrogen phosphate, sodium phosphate dibasic, tert-butylmethyl ether, methanol (MeOH), acetonitrile (ACN) and the derivatisation reagent, Girard T (GT) hydrazine, used for sample and matrix preparation were purchased from Sigma (Steinheim, Germany); glacial acetic acid (>99.5%), matrices αCHCA and HABA were purchased from Fluka (Buchs, Switzerland); trifluoroacetic acid (TFA, 99%) was from Riedel-de Haën.

Urine samples used in this work were obtained from healthy volunteers from the research team. Volunteer's age ranged between 22–30 years, including both male and female. The research ethical committee from the Science Faculty of Ourense approved the study protocol and all the volunteers gave their consent.

2.2. Apparatus

A model UNIVAPO 100H vacuum concentrator centrifuge (UniEquip, Martinsried, Germany) with a model Unijet II refrigerated aspirator vacuum pump (UniEquip) was used for (i) sample drying and (ii) sample pre-concentration. A Spectrafuge-mini minicentrifuge (Labnet, Madrid, Spain) and a Sky Line minicentrifuge-vortex (ELMI, Riga, Latvia) were used throughout the sample treatment, when necessary. A Simplicity 185 system (Millipore, Milan, Italy) was used to obtain Milli-Q ultrapure water throughout all the experiments. The derivatisation procedure was performed in a 1.5 mL microtube flat cap from Delta Lab (Barcelona, Spain). Separation of the steroid Girard T (GT) hydrazones from the unreacted Girard T reagent was carried out in a 2 mL empty reversible solid-phase extraction (SPE) cartridge from Supelco (Belefonte, PA, USA) packed with a preparative C18 resin (125 Å, 55–105 μm; Waters, Barcelona, Spain).

2.3. Sample preparation

2.3.1. Standard solutions

Individual stock standard solutions of each compound (500 mg/L) were prepared by weighing 0.0125 g of analyte in a 25 mL volumetric flask and making it to volume with methanol. These standard solutions were stored in the dark at −20 °C. Working standard solutions were prepared by dilution of the stock standard solutions in the appropriate volume of methanol.

2.3.2. Urine hydrolysis procedure

Urine samples (2 mL) were hydrolysed with 50 μL of the commercial solution of β-glucuronidase, after the addition of 0.750 mL of phosphate buffer (0.8 M, pH 7). The hydrolysis was performed at 55 °C during 60 min.

2.3.3. Liquid–liquid extraction of target analytes

After cooling to room temperature, 0.5 mL of carbonate buffer (pH 9) was added to alkalinize the hydrolyzed solution. Liquid–liquid extraction was carried out by agitation with 5 mL of tert-butyl methyl ether for around 10 min; after centrifugation, the organic phase layer was transferred to a new vessel.

2.3.4. Derivatisation procedure

The procedure for derivatisation with Girard T hydrazine was performed based on the protocol described by Wheeler [29], as follows: The collected organic phase was dried under a gentle nitrogen stream at 40 °C. After the addition of 500 μL of a methanolic solution with 10% glacial acetic acid and 4 mg of Girard T hydrazine, the vial was closed and the derivatisation reaction was then performed at 60 °C during 30 min. After cooling, the solution was evaporated to dryness in a vacuum concentrator centrifuge and then reconstituted with 1 mL of methanol/water (10:90, v/v).

2.3.5. SPE clean-up

After derivatisation, the steroid GT hydrazones were separated from un-reacted GT hydrazine reagent by SPE in a C18 cartridge, according to the protocols described by Khan et al. [24] and Griffiths et al. [25]. Briefly, before use, the cartridges were conditioned with 5 mL of methanol plus 10 mL of MilliQ-water without allowing the cartridges to dry out. After loading the sample, the cartridge was washed with 2 mL of methanol/water (10:90, v/v) in order to remove impurities from the cartridge and, finally, the steroid GT hydrazones were eluted from the cartridge with 1 mL of methanol.