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Screening for anabolic steroids in sports: Analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry☆

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ABSTRACT

In order to improve the detection capabilities of anabolic androgenic steroids (AAS) in sports, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) screening method for the simultaneous detection of AAS phase I and phase II intact urinary metabolites (glucuronides and sulfates) was developed.

A total of 36 metabolites (7 unconjugated; 19 glucuronides and 10 sulfates) corresponding to 15 of the most reported AAS were included. Analytes were extracted from urine using C18 cartridges. LC and MS conditions were studied in-depth to determine the most sensitive and selective conditions for each analyte. A selected reaction monitoring method was set up. The optimization of the experimental parameters for 13 metabolites not available as standards was performed using excretion study urines.

Extraction recoveries were above 77% for all 23 validated analytes. Intra-day precision was lower than 21%, and LODs were in the range 0.25–4 ng/mL for 18 of the 23 analytes. Matrix effect was evaluated using post column infusion and ranged from 92 to 147%. The method was successfully applied to excretion study urines of different exogenous AAS. The suitability of the strategy was demonstrated with methyltestosterone and stanozolol excretion study urines by achieving detection times of 22 and 21 days, respectively.

The method is compliant with the World Antidoping Agency requirements for most of the studied compounds. It represents a cost-effective approach that improves the detection capabilities of AAS by increasing the sensitivity for some metabolites and by including recently described phase II long-term metabolites not detectable using the current screening strategy.

1. Introduction

Anabolic androgenic steroids (AAS) are prohibited in sports due to their performance enhancing properties. They are the most frequently reported group of prohibited substances detected in doping controls, reflecting the wide use of these drugs among athletes [1]. There is a need to continuously improve the detection capabilities of the AAS misuse. The best markers of the administration are the metabolites detected for long time in urine, the so-called long-term metabolites.

Most AAS are extensively metabolized and they are mainly excreted in urine as phase II metabolites [2–4]. However, for many AAS most of the metabolic profile remains unknown [2–15]. Studies on phase II steroid metabolism have been traditionally performed using gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–tandem mass spectrometry (LC–MS/MS), after hydrolysis to release the phase I metabolites [2–4,9–11]. Since most studies used preparations with β-glucuronidase enzymes or preparations with β-glucuronidase and arylsulfatase activities, that show low efficacy to cleave most of the steroid sulfates [16], only glucuronoconjugated metabolites hydrolyzable in these conditions and free excreted metabolites have been systematically studied.

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In recent years, new phase II metabolites have been identified for some AAS using their direct analysis by LC–MS and some of them have shown to be useful as long-term metabolites. Boldenone, boldione, methyltestosterone and metandienone metabolites conjugated with sulfate have been recently identified [5–8,12]. Glucuronoconjugated metabolites poorly or not hydrolyzable using β-glucuronidases have been described for testosterone and stanozolol [13,14]. These results demonstrate the need to re-evaluate AAS metabolism to study new phase II metabolites not systematically studied up to now, to look for new long-term metabolites, and to include these new metabolites in the routine screening procedures.

Current screening methods for AAS are based on the hydrolysis of the urine using β-glucuronidase enzymes, and analysis using both GC–MS and LC–MS technologies [17,18]. Therefore, only unconjugated metabolites and hydrolyzable glucuronic acid conjugates are detectable. Moreover, it is known that the time required to complete the hydrolysis varies between the different steroid glucuronides [19,20] and the hydrolysis may also be incomplete in particular urine matrices due to enzyme inhibition [21]. As a consequence, some metabolites may be underestimated due to incomplete hydrolysis. Furthermore, the current procedure is time consuming and requires the combination of two technologies to ensure detection of all compounds: the need for derivatization before GC–MS analysis limits the analysis of some phase I metabolites that do not form suitable derivatives [18]; and, other phase I metabolites cannot be detected using LC–MS due to the lack of ionizable groups [22].

Steroid glucuronides and sulfates are readily ionized using electrospray ionization [12,13,18,23,24], and methods describing the direct analyses of AAS conjugates by LC–MS have been published although the lack of reference materials has been often a drawback [23,25,26]. Recently, a screening method using liquid chromatography–high resolution mass spectrometry (LC–HRMS) has been described for exogenous AAS [27]. The method was mainly based on the detection of AAS metabolites monitored in the currently used screening strategies and, for some of the compounds, the required limits of detection were not achieved [28].

The aim of the present study was to develop a comprehensive screening method consisting of the analysis of unaltered phase I and phase II metabolites of exogenous AAS by LC–MS/MS. Recently described long-term metabolites as well as the metabolites monitored in the currently used screening procedure were incorporated. The suitability of the method to detect all type of metabolites (unconjugated, glucuronides and sulfates) was evaluated by using excretion study samples of different AAS.

2. Materials and methods

2.1. Chemical and reagents

17β-Nandrolone 17-sulfate (17β-NAN-S) and oxandrolone (OXA) were obtained from Steraloids (Newport, USA). 17α-Methyl-5α-androstan-3α,17β-diol (METm1), 17α-methyl-5β-androstan-3α,17β-diol (METm2), 5α-androstane-3α, 17β-diol 17-glucuronide, 5α-androstane-3α, 17β-diol 3-glucuronide, 5β-androstane-3α, 17β-diol 17-glucuronide, 5β-androstane-3α, 17β-diol 3-glucuronide, 5α-androstane-3β, 17β-diol 3-glucuronide, 5β-androst-1-ene-17β-ol-3-one 17-glucuronide (BOLDm-G), 5β-androstan-7β,17α-dimethyl-3α,17β-diol 3-glucuronide (CALm-G), bolasterone 3-glucuronide (BOLm-G), 17β-boldenone 17-glucuronide (BOLD-G), 17β-boldenone 17-sulfate (BOLD-S), epimetandienone (MEDm2), epioxandrolone (OXAm), 9-fluoro-18-nor-17,17-dimethyl-4,13-diene-11-ol-3-one (FLUm3), 3′-hydroxystanozolol 3′-glucuronide (3STAN-G), 6β-hydroxy-metandienone (MEDm1), 6β-hydroxy-4-chloro-metandienone (4CMDm), 1-methylen-5α-androstan-3α-ol-17-one 3-glucuronide (MTNm-G), 1α-methyl-5α-androstan-3α-ol-17-one 3-glucuronide (MESm1-G), 1α-methyl-5α-androstan-3α-,17β-diol 3-glucuronide (MESm2-G), 2α-methyl-5α-androstan-3α-ol-17-one 3-glucuronide (DROm-G), 17α-nandrolone 17-sulfate (17α-NAN-S), 17β-nandrolone 17-glucuronide (17β-NAN-G), 19-norandrosterone 3-glucuronide (NA-G), 19-norandrosterone 3-sulfate (NA-S), 19-noretiocholanolone 3-glucuronide (NE-G) and 19-noretiocholanolone 3-sulfate (NE-S) were supplied by NMI Australian Government (Pymble, Australia). Methyltestosterone (MET), from Toronto Research Chemicals (Toronto, Canada), and, androsterone-d4 3-glucuronide (d4-And-G), nandrolone-d3 17-sulfate (d3-NAN-S) and testosterone-d3 17-glucuronide (d3-T-G), from NMI Australian Government (Pymble, Australia), were used as internal standards (IS).

Acetonitrile (ACN) (LC gradient grade) and methanol (MeOH) (LC grade), formic acid (LC/MS grade) and ammonium formate were obtained from Merck (Darmstadt, Germany). Acetobromo-α-D-glucuronic acid methyl ester (α-D-Glucopyranuronic acid, 1-bromo-1-deoxy-, methyl ester, 2,3,4-triacetate), toluene, Ag_2CO_3 and LiOH were purchased from Sigma–Aldrich Química S.A. (Madrid, Spain). Milli Q quality water was used (Millipore Ibérica, Barcelona, Spain). Sep-Pak Vac RC C18 (500 mg) cartridges were purchased from Waters (Milford, Massachusetts, USA).

2.2. Sample preparation

After the addition of 20 μL of the Internal Standards (IS) solution (MET, d3-NAN-S and d3-T-G at 1 μg/mL, and d4-And-G at 5 μg/mL), urine samples (2 mL) were vortex-mixed and passed through a C18 cartridge previously conditioned with MeOH (2 mL) and water (2 mL). The column was then washed with water (2 mL), and the analytes were eluted with MeOH (2 mL). The samples were evaporated to dryness under nitrogen stream in a bath at 40 °C. The extract was re-dissolved into 200 μL of a solution of ACN:water (10:90, v/v). A volume of 10 μL was injected into the LC–MS/MS.

2.3. LC–MS/MS instrumental conditions

Detection was carried out using a triple quadrupole (XEVO TQMS) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (all from Waters-Corporation, Milford, MA, USA). Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set to 1200 L/h, and the cone gas flow was 50 L/h. The nitrogen desolvation temperature was 450 °C, and the source temperature was 120 °C.

Chromatographic separations were carried out on an Acquity UPLC® system (Waters Corporation) using an Acquity UPLC® BEH C18 column (2.1 mm × 100 mm i.d., 1.7 μm particle size). The column temperature was set to 45 °C and the flow rate was 0.3 mL/min.

In order to optimize the mobile phase composition, four different mobile phases (MP-1 to MP-4) were studied: MP-1: 0.01% aqueous formic acid (solvent A) and 0.01% formic acid in ACN (solvent B); MP-2: 0.01% aqueous formic acid (solvent A) and 0.01% formic acid in MeOH (solvent B); MP-3: 0.01% formic acid and 1.0 mM ammonium formate in water (solvent A) and 0.01% formic acid and 1.0 mM ammonium formate in ACN:water (95:5, v/v) (solvent B); and MP-4: 0.01% formic acid and 1.0 mM ammonium formate in water (solvent A) and 0.01% formic acid and 1.0 mM ammonium formate in MeOH (solvent B). The same gradient program was used in all cases. The percentage of organic solvent (solvent B) was linearly changed as follows: 0 min, 20%; 2 min, 20%;

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