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 hydrolysable in these conditions and free excreted metabolites have been systematically studied.

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 hydrolysable 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 ºC. The extract was re-dissolved into 200 µL of a solution of ACN:water (10:90, v/v). A volume of 10µL 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 ºC, and the source temperature was 120 ºC.

Chromatographic separations were carried out on an Acquity UPLC® system (Waters Corporation) using an Acquity UPLC® BEH C18 column (2.1 mm x 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%; 15 min, 40%; 16 min, 70%; 17 min, 95%; 18 min, 95%; 18.5 min, 20%; 20 min, 20%.

Mass spectrometric conditions were optimized by infusing methanolic solutions of the compounds. For steroid metabolites not available as standards, optimization was performed using extracts from urines obtained after administration of the AAS. Capìllary voltage and cone voltage were optimized to obtain maximum signal for the parent ions. Collision energies were studied in order to obtain the maximum response for the product ions. For the final screening method, a selected reaction monitoring (SRM) method using retention windows was set up. The two most specific transitions for each compound were selected by evaluation of the signal intensity and the urine background in 10 different blank urines spiked with the metabolites at different concentrations (0.5, 1, 2, 5, 10 and 50 ng/ml). The mass spectrometric conditions of the SRM method are described in Table 1. Switching polarity was used throughout the runs.

2.4. Method validation

The method was validated for qualitative purposes according to the World Antidoping Agency (WADA) requirements using a previously described protocol [29].

The limit of detection (LOD) was evaluated by using decreasing concentrations of the analytes in 10 different urines. The LOD was defined as the lowest concentration at which an analyte was detected in all 10 urine samples with a signal-to-noise ratio of at least 3.

Within-day precision was assessed by analysis of 6 replicates of a urine sample on the same day spiked at two concentrations levels: low (LCL) and upper (UCL). The LCL was the LOD and the UCL was between 2.5 and 10 fold the LOD. Results were expressed as % relative standard deviation (%RSD) of the area ratios between the analyte and the IS. MET was used as IS for the phase I metabolites, d4-And-G for glucuronides ionizing as $[M+NH_4]^+$, d3-T-G for glucuronides ionizing as $[M+H]^+$, and d3-NAN-S for sulfate conjugates.

Extraction recovery was evaluated at the UCL. A blank urine spiked with the analytes before and after sample preparation was analyzed. The areas obtained in the samples (n=6) spiked before sample preparation were compared with the mean of the areas obtained in samples spiked after sample preparation (n=6), representing 100% of recovery.

Lack of carryover was demonstrated by injecting blank urine extracts immediately after samples spiked at the UCL with all analytes. Carryover was considered negligible if analytes were not detected in the blank urines.

Matrix effect (ME) was assessed by a post column infusion strategy previously described [30,31]. A constant flow (2 µL/min) of a solution of each analyte (10 µg/mL) was added after the chromatographic column while an injection of mobile phase solvent and injections of different extracted blank urines (n=4) were carried out. Signal intensities of the ion transition of each analyte for the mobile phase solvent were given 100% (no ME). Ion suppression or enhancement was calculated in terms of percentage of signal intensity obtained for the blank urine samples in comparison with the mobile phase solvent at the retention time of the analyte. The average and RSD of the ME obtained for the four different urine matrices were calculated.

Selectivity and specificity were studied by the analysis of 10 different blank urine samples obtained from different healthy volunteers. The presence of any interfering substance at the retention time of the compounds of interest and the IS was verified.

2.5. Synthesis of 17α-methyl-5α-androstan-3α,17β-diol glucuronide (METm1-G) and 17α-methyl-5β-androstan-3α,17β-diol glucuronide (METm2-G)

The synthesis of the glucuronides was based on a previously described method [32,33]. Briefly, in 0.5 mL of toluene, 100 µg (0.36 µmol, 1 equivalent) of either 17α-methyl-5α-androstan-3α,17β-diol or 17α-methyl-5β-androstan-3α,17β-diol were added followed by 142 µg (0.36 µmol, 1 equivalent) of acetobromo-α-D-glucuronic acid methyl ester and 500 µg of Ag_2CO_3. The reaction mixture was left under vigorous stirring for 24 h at 75 ºC. Then, the mixture was cooled to room temperature, filtered and evaporated to dryness under

vacuum. The solid residue was dissolved in 0.5 mL of MeOH followed by the addition of 0.5 mL of aqueous LiOH 1 M. After 28 h of stirring at room temperature, the solvents were removed under vacuum.

2.6. Excretion study samples

Urines samples collected before and after administration of AAS to healthy male volunteers were used for this study. Ethical approval was granted by Ethical Committee of our Institute (Consorci Mar Parc de Salut de Barcelona, Spain; 2012/4981) and the Spanish Medicines Agency (EudraCT protocol number 2010-002288-80). All subjects participating in the studies gave their written informed consent. All urine sample were kept frozen at < -20ºC until analysis.

Clinical studies consisted of the oral administration of a single dose of methyltestosterone (10 mg) (3 volunteers), boldenone (20 mg) (1 volunteer), metandienone (5 mg) (4 volunteers) or stanozolol (6 mg) (4 volunteers). Samples were collected before administration and at different times up to 31 days, depending on the study.

In addition, for the validation study, drug-free urine samples were collected from ten healthy volunteers and kept frozen at -20ºC.

3. Results and discussion

3.1. Method optimization

Structures of AAS metabolites included in the study are shown in Figures S-1 and S-2 *supplementary material (supp. mat.)*. A total of 36 metabolites including unconjugated metabolites, glucuronides and sulfates were studied. All AAS phase II metabolites commercially available were selected as target analytes (Figure S-1 *supp. mat.*). Other important metabolites present in unconjugated form in urine were also included in the study (4CMEDm, FLUm3, MEDm1, MEDm2, OXA, OXAm) (Figure S-1 *supp. mat.*). In addition, recently reported long-term metabolites of some exogenous AAS (boldenone, metandienone, methyltestosterone, stanozolol) were included, although their standards were not commercially available (Figure S-2 *supp. mat.*) [6,7,12,14].

Mass spectrometric behavior of steroid phase I and phase II metabolites.

To study the mass spectrometric behavior, AAS metabolites were divided in four groups according to their structural features: **I**, unconjugated steroid metabolites; **II**, glucuronides with Δ^{n}-^{3}CO function or pirazole ring; **III**, glucuronides without Δ^{n}-^{3}CO function; and **IV**, sulfate metabolites (Table 2 and Fig S-3 *supp. mat*). Results obtained regarding ionization and collision induced dissociation (CID) behaviors were in agreement with those previously described [18] and are summarized in Table 2.

All steroid metabolites showed to be readily ionizable. Briefly, in positive mode, unconjugated and glucuronides of group II ionized as $[M+H]^+$ [13,18]. Glucuronides of group III showed the adduct $[M+NH_4]^+$ as the major ion [13,18]. Therefore, the glucuronide group facilitated the ionization of AASs not ionizable as aglycons. In negative mode, all glucuronides formed the ion $[M-H]^-$ resulting from deprotonation of the acidic group [13,18]. Sulfate conjugates also exhibited an abundant $[M-H]^-$ ion [18]. Although positive ionization of sulfates was also evaluated, negative ion mode was used in this study for all sulfates as it always yielded most intense signals.

The CID gave common ions or losses depending on the analytes chemical features (Table 2). Fragmentation of glucuronides showed mainly unspecific ions coming from the glucuronide moiety: m/z 177, 159 and 141 in positive mode and m/z 75, 85 and 113 in negative mode [13,18]. Most AAS sulfates exhibited only an ion at

m/z 97 in negative mode corresponding to hydrogensulfate anion [18,34]. For some compounds, with specific chemical features, other important ions were formed such as the product ion resulting from the neutral loss of 15 Da for metabolites with a highly conjugated A ring (Fig S-3 *supp. mat)*, such as sulfate metabolites of boldenone and methandienone [7,12].

Mobile phase composition.

The ionization efficiency of the analytes depends on the mobile phase composition, and it was optimized. Hence, four different mobile phases were tested (MP-1 to MP-4, see experimental section) using combinations of MeOH and ACN, and modifiers such as ammonium formate. For MP-3, the 5% of water in ACN (solvent B) facilitated the solubility of ammonium formate.

The mass spectrometric behavior of four analytes representing the selected groups of compounds (I, MEDm2; II, BOLDm-G; III, MTNm-G; IV, BOLD-S) was investigated. Urine samples, spiked with these analytes at 100 ng/mL and extracted using the procedure described in experimental section, were analyzed using the different mobile phases. Signal intensities, signal-to-noise ratios and chromatographic resolutions were evaluated (Figure S-4 *supp. mat.*). As expected, mobile phases containing ammonium allowed for the formation of $[M+NH_4]^+$ helping in the ionization of metabolites belonging to group III in positive mode. In contrast, signal in negative ionization mode decreased around two times with this modifier. Although mobile phases containing MeOH provided a superior ionization, those containing ACN gave higher signal-to-noise ratios (Figure S-4(b) *supp. mat.*), less interferent peaks and also better chromatographic resolution regarding pairs of isomers. Therefore, conditions MP-3 were selected to be used in the screening procedure.

Selection of the target ion transitions.

The ion transitions to monitor each steroid metabolite were selected based on the signal intensity and the selectivity. These parameters were evaluated for different ion transitions of each analyte by using ten different urine samples spiked with the analytes at different concentrations. The selection was compound dependent, and ion transitions included in the final SRM method are listed in Table 1. For sulfate conjugates, the ion transitions that yielded the highest signal were those to the product ion m/z 97 and, for most sulfate

metabolites, only these transitions were monitored (Table 1), resulting in high sensitivity and specificity for these compounds. However, for other sulfates with molecular masses closer to endogenous compounds such as BOLD-S, the ion transition $[M-H]^-$ to m/z 97 showed low specificity and ion transitions resulting from characteristic fragmentations (m/z 365→350, m/z 365→177) [12] had to be used to obtain the highest selectivity and adequate LOD (Figure 1 (a)). The poor LOD described for BOLD-S in a recent study using LC-HRMS was due to the presence of endogenous interferences when using either $[M+H]^+$ or $[M-H]^-$ as diagnostic ions [27].

For the majority of glucuronides, the ion transitions were related to the glucuronide moiety and the best signal intensity was in general obtained in negative ionization mode (product ions m/z 75, 85, 113). For 3STAN-G, some ion transitions were strongly interfered by the background, and an in-depth study of the ionization and fragmentation was required. A MS^3–like strategy based on the CID of in-source fragments was used to have more selective transitions. As shown in Figure 1 (b) the SRM transition $[M+H]^+$ (m/z 521) to m/z 345 for 3STAN-G had a big interfering peak that was not present when monitoring the ion transition $[M+H\text{-}gluc]^+$ (m/z 345) to m/z 97. The same strategy was used for some unconjugated compounds, such as MEDm1 (Figure 1 (c)) for which the use of the in source fragment $[M+H-2H_2O]^+$ (m/z 281) as precursor ion improved its detectability compared to the use of $[M+H]^+$ (m/z 317). Except for some sulfates, two ion transitions were selected to monitor each metabolite (Table 1).

3.2. Method validation

The method was validated for qualitative purposes using the AAS metabolites available as reference standards. Results of LOD, extraction recovery, repeatability at two concentrations levels and ME are listed in Table 3.

The method showed to be selective and specific, as no matrix interferences were detected at the retention times of the compounds of interest in blank urine samples. The chromatograms obtained after analysis of a representative blank urine sample are shown in Figure S-5 (*supp. mat.*). Blank samples injected after samples spiked at the high concentration level did not present any traces of carryover.

The extraction recoveries were better than 77% and, therefore, the sample pre-treatment procedure was appropriate to recover free as well as conjugated metabolites. As for the current methods to detect AASs, a substantial reduction of time in sample preparation procedure was achieved due to the elimination of the hydrolysis and derivatisation procedures.

Good intra-assay precision was obtained, with RSD values within the normally accepted ranges, lower than 15% for the UCL studied and lower than 25% for the LCL.

Detection of the analytes can be affected by ion suppression or enhancement due the impact of coeluting substances of the urine matrix. Results showed moderate enhancement (from 104 to 147 %) of the analytes ions intensities except for FLUm3 that showed small suppression (Table 3). RSD values were lower than 10%, indicating similar behavior between the different urine matrices.

A detail of the results obtained in the evaluation of the LOD is shown in Table S-6 (*supp. mat.*) and results are summarized in Table 3. The LODs obtained ranged from 0.25 to 4 ng/mL for free metabolites, from 0.25 to 0.5 ng/mL for sulfate conjugates and from 0.5 to more than 20 ng/mL for glucuronide conjugates. The LOD required by WADA for AAS metabolites is 2.5 ng/mL of the phase I metabolite except for dehydrochlormethyltestosterone, methandienone, methyltestosterone and stanozolol metabolites for which the required LOD is 1 ng/mL [28]. Taking into consideration that a concentration of 2.5 ng/mL of the phase I metabolite is equivalent to approximately 4 and 3 ng/mL of the glucuronides and sulfates, respectively, the LODs obtained for most of the compounds are in agreement with WADA requirements.

Five glucuronides (MESm1-G, 17β-NAN-G, BOLDm-G, CALmG and BOLmG) and two unconjugated (MEDm1 and 4CMEDm) metabolites showed LODs greater than those required (Table 3). For MESm1-G, 17β-NAN-G and BOLDm-G (metabolites of mesterolone, nandrolone and boldenone respectively), other metabolites of these AAS are included in the procedure with good LOD (MESm2-G for mesterolone; NE-G, NA-G, NE-S and NA-S for nandrolone; and BOLD-G, BOLD-S and eBOLD-S for boldenone). Therefore, the detection capabilities of these AAS are not compromised. Similarly, the relatively high LOD of MEDm1 does not compromise the detection of metandienone misuse because other metabolites, including a recently described long-term metabolite, MEDm-S, are also monitored [7] (Figure S-2 *supp. mat.*). 4CMEDm is a

metabolite present in urine during the first days after administration of dehydrochlormethyltestosterone, and the improvement in the detection capabilities for that AAS has to be achieved by monitoring the recently described long-term metabolites excreted as glucuronides [9]. Finally, the poor detection of CALm-G and BOLm-G suggests the need to perform metabolic studies of calusterone and bolasterone to look for alternative metabolites to be included in the method in order to improve the detection of these AAS using the proposed procedure.

Previous works involving the detection of five intact AAS glucuronide metabolites [35] showed LODs in the range 5-10 ng/mL. A recently published method using urine dilution and LC-HRMS analysis [27] showed higher LODs for all unconjugated and sulfate metabolites except for OXAm for which the same LOD was obtained. Regarding glucuronide metabolites, LODs were from 2 to 26 fold higher with the exception of CALm-G and BOLm-G. These results indicate the importance of using a sample preparation procedure and more selective ion transitions, as in our procedure, to achieve the required sensitivity.

Results of one of the blank urine samples spiked with the 23 studied compounds at their LODs are shown in Figure S-7 (*supp. mat.*).

3.3. Inclusion of recently described long-term metabolites of some AAS

New long-term phase II metabolites have been recently described for some AAS (e.g., boldenone, metandienone, methyltestosterone and stanozolol), for which standards are not commercially available [6,7,12,14]. For this reason, urines obtained after administration of these AAS were used to optimize the analysis conditions of the metabolites and to incorporate them into the screening procedure. The structures of the metabolites are shown in Figure S-2 (*supp. mat.*) and their analytical conditions are listed in Table 1. Metandienone and boldenone sulfate metabolites, MEDm-S [7] and eBOLD-S [5,12], were included. For stanozolol, three recently reported glucuronides STAN-*N*-G, STAN-*O*-G and eSTAN-*N*-G, together with 4STAN-G and 16STAN-G were incorporated to the method [14]. Regarding methyltestosterone, glucuronides METm1-G and METm2-G, unconjugated metabolite METm5 [15] and the recently described long-term metabolites METm2-S, METm3-S and METm4-S [6] were included. METm1-G and METm2-G are the

conventional metabolites detected in the current screening procedures as the corresponding phase I metabolites [2,3].

Due to the importance of METm1-G and METm2-G in the currently used screening procedures and in WADA requirements [28], these metabolites were qualitatively synthesized and the analysis revealed the occurrence of one peak for METm1-G and two peaks for METm2-G corresponding to the 3-/17-glucuronide isomers. The identification of these isomers (Table 1) was based on the comparison of chromatographic and mass spectrometric data with data obtained for androstanediol metabolites which have a similar structure and the four possible metabolites commercially available (data not shown).

3.4. Excretion studies of methyltestosterone and stanozolol.

The method was tested using samples collected after administration of the AAS to volunteers. As an example of the usefulness and capabilities of the developed method for screening purposes, results of the analysis of methyltestosterone and stanozolol excretion urine samples are presented. Figure 2 shows representative chromatograms of the simultaneous detection of six different methyltestosterone metabolites. The longest detection time was obtained with METm3-S. Detection of sulfate metabolites (METm2-S, METm3-S and METm4-S) [6] was possible up to 8, 22 and 11 days, respectively, which is in agreement with previously reported detection times for these metabolites using other analytical approaches [6]. As mentioned, so far these sulfate metabolites are not monitored in the current screening methods used in most laboratories.

Detection of the unconjugated metabolite METm5 is around one week, in accordance with previously reported data using LC-MS/MS analysis [6]. Furthermore, detection of unaltered glucuronide metabolites METm1-G and METm2-3-G was up to 1 and 4 days, respectively. Using GC-MS/MS analysis after hydrolysis and derivatization, detection times of 4 and 6 days were described for METm1-G and METm2-G, respectively [6,36]. The developed methodology could distinguish the two different METm2-G isomers (METm2-3-G and METm2-17-G) which are observed in only one peak (corresponding to the phase I metabolite) using the GC-MS/MS procedure. This could partially explain the differences in detection times observed for METm2-G. However, the main reasons for these differences are the high sensitivity of GC-MS/MS analysis for the phase I metabolites, METm1 and METm2, and a low sensitivity for the glucuronides METm1-G and METm2-G in

LC-MS/MS, that need to be ionized through the formation of the adduct ion $[M+NH_4]^+$ due to the lack of ionizable groups in the steroid structure.

Results show that although the detection times obtained for some metabolites are lower (and probably the LOD higher) than using conventional screening methods, the capabilities of the detection of methyltestosterone misuse have been improved using the proposed procedure from 6 to 22 days after administration, through the monitoring of new long-term metabolites. It is worth mentioning that the proposed procedure allows for the detection of different types of metabolites (unconjugated metabolites and conjugates with glucuronic acid or sulfate) that need three different methods using the conventional approaches [6].

Results obtained after analysis of stanozolol samples are shown in Figure 3. Six different glucuronides were monitored (4STAN-G, 16STAN-G, 3STAN-G, STAN-*N*-G, STAN-*O*-G and eSTAN-*N*-G), and they were detected up to 4, 7 ,12 ,5 , 4 and 21 days, respectively. The detection time for eSTAN-*N*-G is in accordance with recently published data [14]. As described for methyltestosterone, the detection capabilities for stanozolol have been significantly improved compared with current screening methods through the monitoring of recently reported long-term metabolites. For stanozolol metabolites, a more selective sample preparation procedure based on cation exchange solid phase extraction taking advantage of its basic nitrogen may be used to obtain cleaner extracts for confirmation purposes as previously described [37].

4. Conclusions

A LC-MS/MS screening method for the simultaneous detection of phase I and phase II intact urinary metabolites of exogenous AAS was developed and fully validated for the first time. The validation criteria were met and the method was considered compliant with WADA requirements for most of the analytes. Moreover, it represents a much simple approach with a less time-consuming sample preparation compared to what conventional LC-MS and GC-MS methods require. Automatization of the procedure with an on-line solid phase extraction system could be easily implemented in the future. In addition, the method improves the detection capabilities through the monitoring of important long term metabolites not detectable using the current screening strategy and, also, it could incorporate new long-term phase II metabolites described in the future irrespective of their nature. Endogenous AAS metabolites could be, as well, included in the method. In order to demonstrate the suitability of the strategy methyltestosterone and stanozolol excretion study urines were analysed reaching detection times of 22 and 21 days, respectively. The method can readily be incorporated to WADA accredited laboratories as it offers a direct and sensitive approach to the analysis of exogenous steroids.

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Appendix A. supplementary data

Supplementary data associated with this article can be found in the online version.

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Table 1. Compounds with available and non-available reference standard included in the screening method: retention time (RT), ionization mode (POS, positive; NEG, negative), precursor ion, cone voltages (CV), collision energies (CE) and product ions. The SRM transitions in bold were used for validation.

Table 2. Summary of the MS behavior of the AAS metabolites divided in four groups according to the structure.

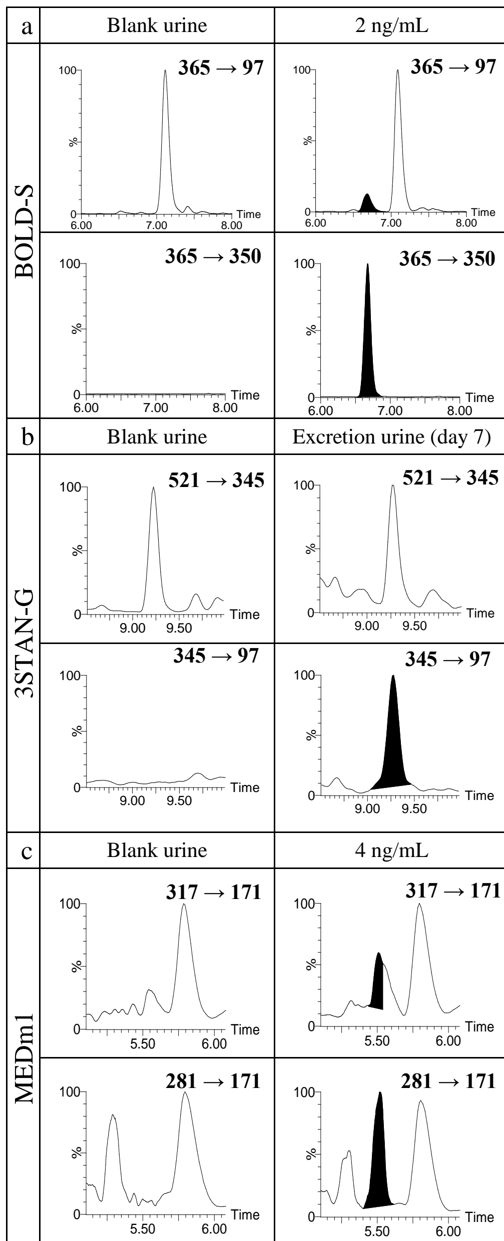
Figure 1. Differences in the detection of BOLD-S (a), 3STAN-G (b) and MEDm1 (c) in urine using the ion transitions involving common ions/losses (top) and using specific transitions or in-source fragments (bottom).

Table 3. LOD, extraction recovery, within assay precision and matrix effect results of the validation for qualitative purposes for the commercially available compounds.

Figure 2. Results of an administration study of methyltestosterone: chromatograms of the characteristic ion transitions of the metabolites METm2-3-G, METm1-G, METm5, METm2-S, METm3-S and METm4-S, obtained after analysis of a pre-administration sample (day 0) and samples collected at 1, 4, 7, 11 and 22 days after methyltestosterone administration.

Figure 3. Results of an administration study of stanozolol: chromatograms of the characteristic ion transitions of the metabolites 4STAN-G, 16STAN-G, 3STAN-G, STAN-N-G, STAN-O-G and eSTAN-N-G, obtained after analysis of a pre-administration sample (day 0) and samples collected at 4, 5, 12 and 21 days after stanozolol administration.

Figure 1. Differences in the detection of BOLD-S (a), 3STAN-G (b) and MEDm1 (c) in urine using the ion transitions involving common ions/losses (top) and using specific transitions or in-source fragments (bottom).



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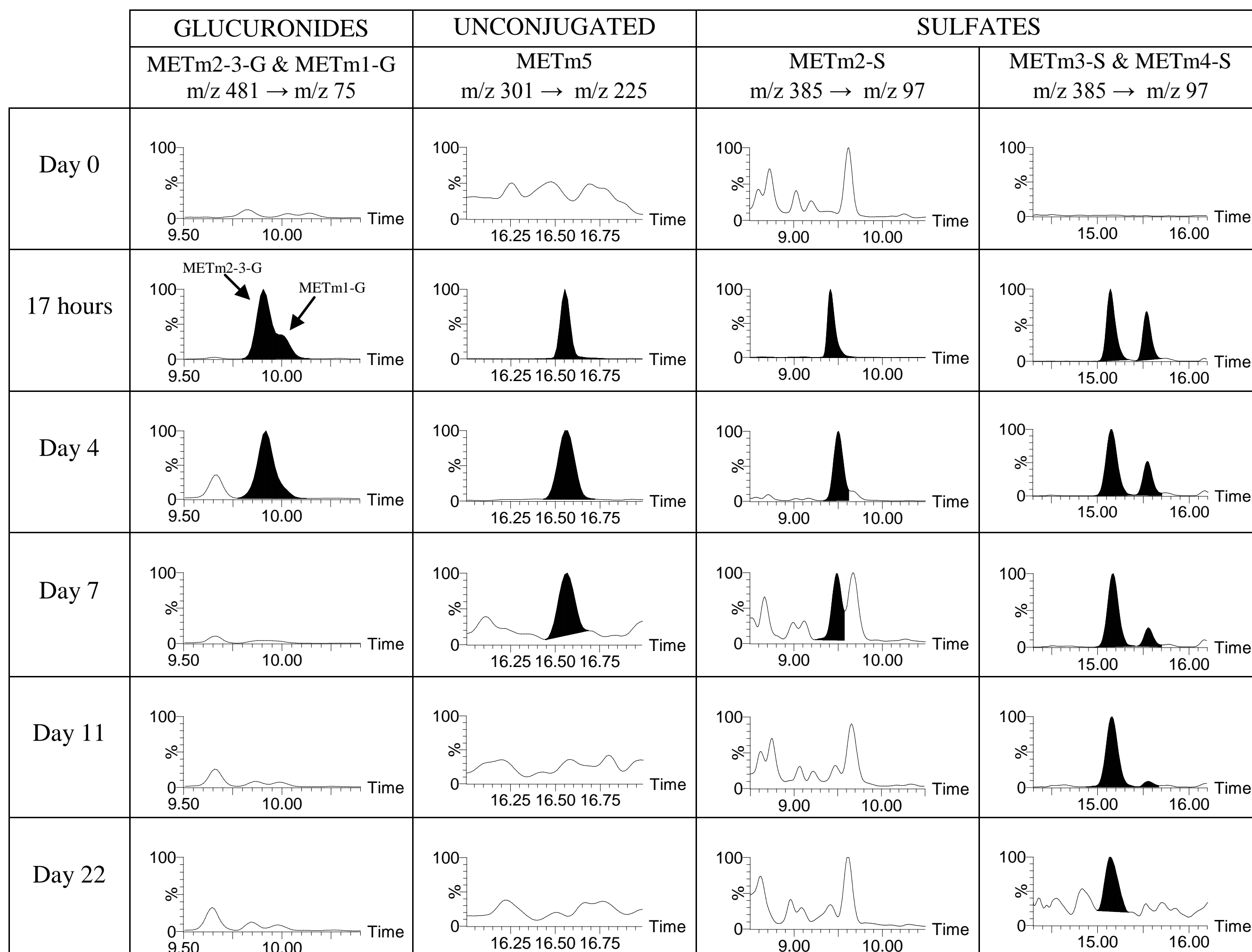

Figure 3. Results of an administration study of stanozolol: chromatograms of the characteristic ion transitions of the metabolites 4STAN-G, 16STAN-G, 3STAN-G, STAN-N-G, STAN-O-G and eSTAN-N-G, obtained after analysis of a pre-administration sample (day 0) and samples collected at 4, 5, 12 and 21 days after stanozolol administration.

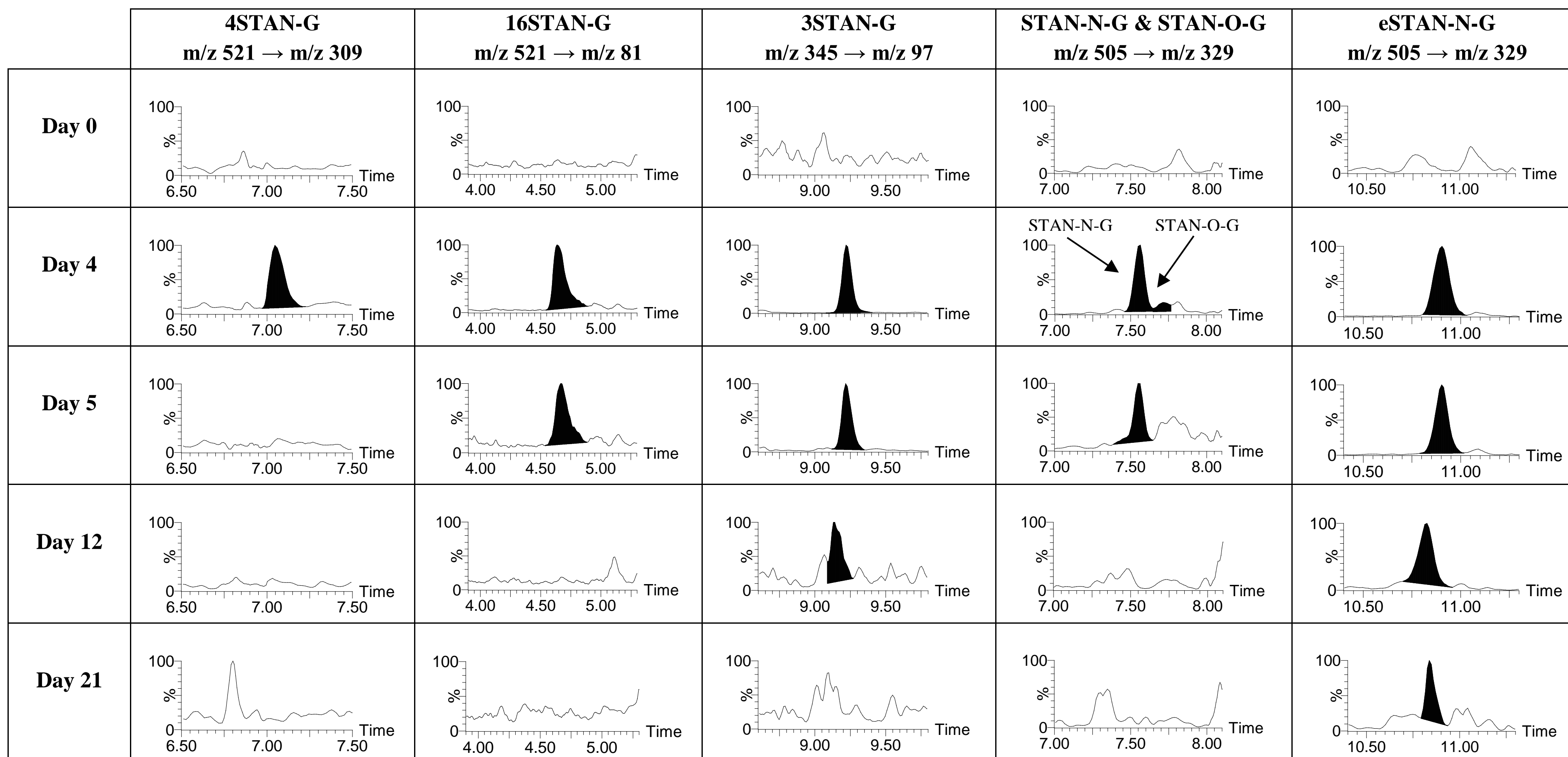

Table 1. Compounds with available and non-available reference standard included in the screening method: retention time (RT), ionization mode (POS, positive; NEG, negative), precursor ion, cone voltages (CV), collision energies (CE) and product ions. The SRM transitions in bold were used for validation.

		Compound *		RT (min)	Mode (ESI)	Precursor Ion (m/z)		CV (V)	CE (eV)	Product Ion (m/z)
Compounds with available reference standard	Unconjugated	MEDm1	6β-hydroxy-metandienone	5,58	**POS**	**281**	**$[M+H-2H_2O]^+$**	**30**	**30**	**171**
					POS	299	$[M+H-1H_2O]^+$	25	25	121
		4CMEDm	6β-hydroxy-4-chloro-metandienone	9,00	**POS**	**351**	**$[M+H]^+$**	**15**	**20**	**147**
					POS	351	$[M+H]^+$	15	50	91
		OXA	oxandrolone	12,68	**POS**	**307**	**$[M+H]^+$**	**20**	**30**	**93**
					POS	307	$[M+H]^+$	25	10	289
		OXAm	epioxandrolone	16,22	**POS**	**307**	**$[M+H]^+$**	**20**	**30**	**93**
					POS	307	$[M+H]^+$	20	10	289
		MEDm2	epimetandienone	16,42	**POS**	**301**	**$[M+H]^+$**	**20**	**15**	**149**
					POS	301	$[M+H]^+$	20	10	283
		FLUm3	9-fluoro-18-nor-17,17-dimethyl-4,13-diene-11-ol-3-one	17,02	**POS**	**319**	**$[M+H]^+$**	**35**	**25**	**225**
					POS	319	$[M+H]^+$	35	25	281
	Sulfates	17β-NAN-S	17β-nandrolone 17-S	6,04	**NEG**	**353**	**$[M-H]^-$**	**55**	**35**	**97**
		BOLD-S	17β-boldenone 17-S	6,61	**NEG**	**365**	**$[M-H]^-$**	**55**	**30**	**350**
					NEG	365	$[M-H]^-$	55	40	177
		17α-NAN-S	17α-nandrolone 17-S	6,88	**NEG**	**353**	**$[M-H]^-$**	**55**	**35**	**97**
		NA-S	19-norandrosterone 3-S	9,67	**NEG**	**355**	**$[M-H]^-$**	**55**	**35**	**97**
		NE-S	19-noretiocholanolone 3-S	9,70	**NEG**	**355**	**$[M-H]^-$**	**55**	**35**	**97**
	Glucuronides	17β-NAN-G	17β-nandrolone 17-G	5,91	**POS**	**451**	**$[M+H]^+$**	**30**	**30**	**85**
					NEG	449	$[M-H]^-$	45	30	113
		BOLD-G	17β-boldenone 17-G	6,16	**POS**	**463**	**$[M+H]^+$**	**20**	**25**	**121**
					POS	463	$[M+H]^+$	20	20	135
		3STAN-G	3'-hydroxystanozolol 3'-G	9,22	**POS**	**345**	**$[M+H-gluc]^+$**	**60**	**45**	**97**
					NEG	519	$[M-H]^-$	45	30	343
		BOLDm-G	5β-androst-1-ene-17β-ol-3-one 17-G	9,65	**NEG**	**463**	**$[M-H]^-$**	**30**	**40**	**85**
					POS	465	$[M+H]^+$	25	25	187
		NE-G	19-noretiocholanolone 3-G	10,20	**POS**	**470**	**$[M+NH_4]^+$**	**20**	**25**	**241**
					POS	470	$[M+NH_4]^+$	20	20	259
		MESm2-G	1α-methyl-5α-androstan-3α-,17β-diol 3-G	10,28	**NEG**	**481**	**$[M-H]^-$**	**50**	**35**	**75**
					NEG	481	$[M-H]^-$	50	35	85
		BOLm-G	bolasterone 3-G	10,55	**NEG**	**495**	**$[M-H]^-$**	**50**	**45**	**85**
					POS	514	$[M+NH_4]^+$	15	40	175
		NA-G	19-norandrosterone 3-G	10,68	**NEG**	**451**	**$[M-H]^-$**	**50**	**35**	**85**
					NEG	451	$[M-H]^-$	50	35	75
		MTNm-G	1-methylen-5α-androstan-3α-ol-17-one 3-G	11,47	**NEG**	**477**	**$[M-H]^-$**	**50**	**35**	**75**
					POS	496	$[M+NH_4]^+$	15	15	285
		CALm-G	5β-androstan-7β,17α-dimethyl-3α,17β-diol 3-G	11,80	**NEG**	**495**	**$[M-H]^-$**	**50**	**45**	**75**
					NEG	495	$[M-H]^-$	50	45	85
		MESm1-G	1α-methyl-5α-androstan-3α-ol-17-one 3-G	12,95	**NEG**	**479**	**$[M-H]^-$**	**55**	**35**	**85**

					NEG	479	$[M-H]^-$	55	35	75
		DROm-G	2α-methyl-5α-androstan-3α-ol-17-one 3-G	14,79	**NEG**	**479**	**$[M-H]^-$**	**55**	**35**	**85**
					NEG	479	$[M-H]^-$	50	35	75
Compounds with non-available reference standard **	Unconj	METm5	17α-hydroxy-17β-methylandrost-4,6-dien-3-one	16,57	POS	301	$[M+H]^+$	25	20	225
					POS	301	$[M+H]^+$	25	30	210
	Sulfates	eBOLD-S	epiboldenone 17-S	7,32	NEG	365	$[M-H]^-$	55	30	350
					NEG	365	$[M-H]^-$	55	40	177
		MEDm-S	18-nor-17β-hydroxymethyl-17α-methylandrost-1,4,13-triene-3-one 18-S	7,52	NEG	377	$[M-H]^-$	60	40	80
					NEG	377	$[M-H]^-$	60	30	362
		METm2-S	17α-methyl-5β-androstan-3α,17β-diol 3-S	9,47	NEG	385	$[M-H]^-$	60	40	97
		METm3-S	17β-methyl-5α-androstan-3α,17α-diol 3-S	15,16	NEG	385	$[M-H]^-$	60	40	97
		METm4-S	17β-methyl-5β-androstan-3α,17α-diol 3-S	15,56	NEG	385	$[M-H]^-$	60	40	97
	Glucuronides	16STAN-G	16β-hydroxy-stanozolol 16-G	4,64	POS	521	$[M+H]^+$	25	65	81
					POS	521	$[M+H]^+$	25	40	345
		4STAN-G	4β-hydroxy-stanozolol 4-G	7,08	POS	521	$[M+H]^+$	25	25	309
					NEG	519	$[M-H]^-$	45	25	193
		STAN-*N*-G	stanozolol-*N*-G	7,55	POS	505	$[M+H]^+$	25	45	329
					POS	505	$[M+H]^+$	25	65	81
		STAN-*O*-G	stanozolol-*O*-G	7,74	POS	505	$[M+H]^+$	25	45	329
					POS	505	$[M+H]^+$	25	65	81
		METm2-3-G METm1-G METm2-17-G	17α-methyl-5β-androstan-3α,17β-diol 3-G 17α-methyl-5α-androstan-3α,17β-diol 3/17-G 17α-methyl-5β-androstan-3α,17β-diol 17-G	9,91 10,01 10,29	POS	500	$[M+NH_4]^+$	15	40	161
						500	$[M+NH_4]^+$	15	20	271
					NEG	481	$[M-H]^-$	50	35	75
						481	$[M-H]^-$	50	35	85
		eSTAN-*N*-G	17-epistanozolol-*N*-G	10.90	POS	505	$[M+H]^+$	25	45	329
					POS	505	$[M+H]^+$	25	65	81
Internal standards		d3-NAN-S	nandrolone-d3 17-S	6,11	NEG	356	$[M-H]^-$	55	40	98
		d3-T-G	testosterone-d3 17-G	7,30	POS	468	$[M+H]^+$	35	25	97
					NEG	466	$[M-H]^-$	45	30	85
		d4-And-G	androsterone-d4 3-G	12,08	POS	488	$[M+NH_4]^+$	15	40	95
					NEG	469	$[M-H]^-$	50	35	85
		MET	methyltestosterone	14,81	POS	303	$[M+H]^+$	30	25	109

* S: sulfate; G:glucuronide

** Extracts from urines obtained post-administration of parent compounds have been used

Table 2. Summary of the MS behavior of the AAS metabolites divided in four groups according to the structure.

Group	Compounds	Conjugation	A ring	Ionization	Species	Fragmentation	
						Product ions (m/z)	Neutral losses (Da)
I	FLUm3	unconjugated	Δ^n-^{3}CO	ESI +	$[M+H]^+$	Compound dependent	-
	6βOH-4CMED						
	MEDm1						
	MEDm2			ESI -	-	-	-
	OXA						
	OXAm						
II	BOLD-G	glucuronide	Δ^n-^{3}CO or pirazol	ESI +	$[M+H]^+$	Compound dependent 177, 159, 141	176, 194
	BOLDm-G						
	17β-NAN-G			ESI -	$[M-H]^-$	75, 85, 113	176
	3STAN-G						
III	BOLM-G	glucuronide	^{3}OH	ESI +	$[M+NH_4]^+$	Compound dependent 177, 159, 141	193, 211, 229
	CALm-G						
	DROm-G						
	MESm1-G						
	MESm2-G			ESI -	$[M-H]^-$	75, 85, 113	176
	MTNm-G						
	NA-G						
	NE-G						
IV	BOLD-S	sulfate	Δ^n-^{3}CO or ^{3}OH	ESI -	$[M-H]^-$	97 96, 80 (AAS with 17β-OH)	15 (steroids with conjugated A ring)
	17α-NAN-S						
	17β-NAN-S						
	NA-S						
	NE-S						

Table 3. LOD, extraction recovery, within assay precision and matrix effect results of the validation for qualitative purposes for the commercially available compounds.

	Compound	LOD (ng/mL)	Extraction recovery (%), mean ± SD	Within assay precision				% Matrix effect (RSD %)
				ng/mL	RSD%	ng/mL	RSD%	
Unconjugated	FLUm3	0,25	77,8 ± 12,70	0,25	9,1	2,5	2,3	92 (2,35)
	MEDm2	0,5	85,2 ± 10,7	0,5	11,1	5	1,6	116 (4,31)
	OXA	1	123,9 ± 1,9	1	20,2	5	13,7	116 (4,95)
	OXAm	1	109,9 ± 6,3	1	15,7	5	8,2	109 (1,48)
	4CMEDm	4	92,2 ± 4,6	4	10,6	20	6,1	100 (8,77)
	MEDm1	4	95,7 ± 4,9	4	16,2	20	7,3	112 (8,02)
Sulfates	BOLD-S	0,25	93,2 ± 7,7	0,25	4,5	2,5	9,6	118 (4,35)
	17β-NAN-S	0,25	96,2 ± 1,4	0,25	12,9	2,5	7,2	125 (7,81)
	17α-NAN-S	0,5	87,1 ± 2,1	0,5	8,8	5	4,0	138 (3,07)
	NA-S	0,5	93,8 ± 1,5	0,5	10,8	5	3,8	141 (6,69)
	NE-S	0,5	92,30 ± 9,6	0,5	8,3	5	12,7	114 (6,81)
Glucuronides	NE-G	0,5	77,1 ± 13,5	0,5	19,8	5	18,9	141 (8,50)
	BOLD-G	0,5	93,6 ± 5,2	0,5	21,4	5	7,7	123 (10,09)
	3STAN-G	0,5	87,6 ± 7,1	0,5	9,9	5	5,7	120 (2,13)
	DROm-G	2	86,8 ± 4,0	2	7,8	10	1,6	104 (2,55)
	MESm2-G	4	98,3 ± 3,8	4	5,2	20	12,1	119 (5,30)
	NA-G	4	95,0 ± 6,7	4	10,7	20	9,1	147 (9,19)
	MTNm-G	4	82,8 ± 3,5	4	18,0	20	2,1	144 (5,36)
	MESm1-G	8	88,7 ± 4,4	8	13,1	20	3,8	132 (2,19)
	17β-NAN-G	10	96,0 ± 4,4	10	17,8	50	5,8	115 (5,03)
	BOLDm-G	16	94,9 ± 4,7	16	10,6	40	8,5	112 (4,31)
	CALm-G	20	93,5 ± 2,0	20	18,8	50	6,0	119 (1,78)
	BOLm-G	>20	92,9 ± 1,4	20	12,6	50	1,9	130 (2,61)