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Direct detection and quantification of 19-norandrosterone sulfate in human urine by liquid chromatography-linear ion trap mass spectrometry

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Abstract

19-Norandrosterone sulfate (19-NAS) is the sulfoconjugated form of 19-norandrosterone (19-NA), the major metabolite of the steroid nandrolone. A sensitive and accurate liquid chromatography/tandem mass spectrometry (LC-MS/MS) assay was developed for the direct measurement of 19-NAS in human urine samples. The method involved a quaternary amine SPE protocol and subsequently injection of the extract onto an analytical column (Uptisphere® ODB, 150 mm × 3.0 mm, 5 μm) for chromatographic separation and mass spectrometry detection in negative electrospray ionisation mode. The sulfoconjugate of 19-NA was identified in urine by comparison of mass spectra and retention time with a reference substance. The limit of detection (LOD) and lowest limit of quantification (LLOQ) of 19-NAS were of 40 pg/mL and 200 pg/mL, respectively. For a nominal concentration of 2 ng/mL, recovery (94%), intra-day precision (2.7%), intra-assay precision (6.6%) and inter-assay precision (14.3%) were determined. Finally, this analytical method was applied for quantifying the concentration of 19-NAS in doping samples, using calibration curves (0.2–20 ng/mL) and the standard-addition method. The results show the feasibility of applying this LC-MS/MS assay as a complementary tool to detect misuse of nandrolone or nandrolone precursors.
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Keywords: Nandrolone; 19-Norandrosterone sulfate; Liquid chromatography-mass spectrometry; Doping control

1. Introduction

Nandrolone, or 19-nortestosterone (19-NT), is an anabolic steroid initially introduced for the treatment of anemia, osteoporosis, and breast carcinoma [1,2]. Because of its anabolic properties, nandrolone is used among athletes as a doping agent to accelerate muscle growth; to increase lean body mass, strength and aggressiveness [3]. Detection of nandrolone according to the protocols set down by the World Anti-Doping Agency (WADA) are based on the identification of 19-norandrosterone (19-NA; 3α-hydroxy-5α-androstan-17-one) and 19-noretiocholanolone (19-NE; 3α-hydroxy-5β-androstan-17-one) which are usually the two principal urinary metabolites [4,5]. An adverse analytical finding is reported when the total of free 19-NA and its glucuronide expressed as mass concentration of 19-NA in urine is greater than the threshold of 2 ng/mL established by WADA [6]. This limit has been fixed to take into account possible endogenous production of nandrolone [6,7].

Currently, both 19-NA and 19-NE metabolites are detected in human urine samples following isolation through solid-phase extraction, deconjugation of the glucuronide forms by enzymatic hydrolysis (β-glucuronidase) and derivatization (usually trimethylsilylation) prior to analysis by gas chromatography/mass spectrometry (GC-MS) [8,9]. However, this procedure does not provide detailed information about phase II metabolism that leads to formation of steroid glucuronide and sulfate as main conjugated forms. Nevertheless, the concentration of sulfoconjugates may be obtained indirectly by using deconjugation methods based on acid and enzyme hydrolyses [10–12]. It has been found that nandrolone and other norsteroids administered orally are metabolised into 19-norandrosterone, 19-noretiocholanolone and 19-norepiandrosterone (19-NEA; 3β-hydroxy-5α-androstan-17-one) [10–12]. The first two are predominantly excreted in urine as glucuronide conjugates whereas the last one is almost exclusively sulfoconjugated.

During the last decade, LC-MS/MS methodology has been used for detection of various steroid sulfates in urine samples

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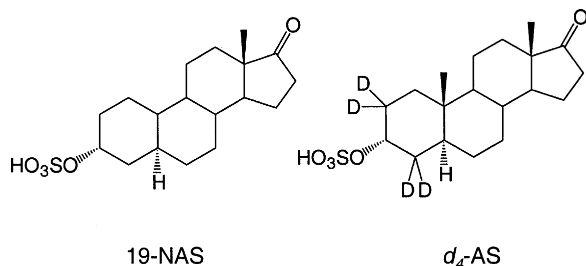

Fig. 1. Structures of 19-norandrosterone sulfate and d_4-androsterone sulfate.

[13–16]. In this study, we propose a sensitive method for the direct measurement of 19-norandrosterone sulfate (19-NAS) concentration in human urine sample (Fig. 1). For that purpose, detection and quantification of (19-NAS) by liquid chromatography tandem mass spectrometry (LC-MS/MS) with a linear ion trap quadripole (LTQ) has been developed. Direct analysis of steroid sulfates by LC-MS provide the advantage of avoiding potential problems caused by incomplete enzyme hydrolysis and incomplete derivatization of the steroids [17,18].

2. Experimental

2.1. Reagents and chemicals

Methanol (99.9%), glacial acetic acid (>99.8%), sodium acetate (>99%) and formic acid (89–91%) were purchased from Merck (Darmstadt, Germany) and sodium hydroxide was obtained from BDH Laboratory Supplies (Poole, England). Water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard® 2 and a Quantum™ EX Ultrapure organex cartridge purchased by Millipore Corp. (Billerica, MA, USA). Bakerbond spe™ 500 mg octadecyl C_{18} disposable extraction columns were obtained from JT Baker (Phillipsburg, NJ, USA) and Bond Elut® SAX 500 mg trimethylaminopropyl from Varian Inc. (Palo Alto, CA, USA). Commercial reference steroids 5α-estran-3α-ol-17-one sulfate triethylammonium salt (19-NAS) and 2,2,4,4-$^2H_4$-5α-androstan-3α-ol-17-one sulfate (d_4-androsterone sulfate, d_4-AS) triethylammonium salt were purchased from NARL (Pymble, Australia).

2.2. Urine sample extraction

The sample extraction is based on a method described first by Vanluchene and Vandekerckhove [19] and subsequently modified by Antignac et al. [20]. An aliquot of urine (1 mL) was spiked with 3 ng d_4-AS as internal standard and 2.0 mL acetate buffer 2 M pH 5.2 was added. Then, sample was extracted on SPE C_{18} disposable extraction column with 500 mg of sorbent, previously conditioned with 5 mL of methanol and 5 mL of water. After washing with 5 mL of water, steroid sulfate compounds were eluted with 10 mL of methanol. Then the extract was submitted to a quaternary ammonium SPE column (Bond Elut SAX) previously conditioned with 3 mL MeOH, 3 mL H_2O, 10 mL acetic acid 3% in water, 10 mL H_2O and 3 mL MeOH. After washing with 6 mL H_2O and 12 mL formic acid 0.75 M in methanol, sulfates were eluted with 20 mL NaOH 1 M adjusted to pH 5.0 with acetic acid. Then, the eluate was applied on SPE C_{18} disposable extraction column with 500 mg of sorbent, previously conditioned with 5 mL of methanol and 5 mL of water. After washing with 10 mL of water, sulfates were eluted with 10 mL of MeOH. Finally, the eluate was evaporated to dryness under a nitrogen stream (Turbo Vap LV evaporator, Zymark, Hopkinton, MA, USA) and then the residue was reconstituted in 100 μL of methanol:1% acetic acid in water (50:50;v/v) prior to LC-MS/MS injection.

2.3. LC-MS/MS conditions

The LC-MS/MS system used for the assay comprised a Rheos 2000 CPS-LC system pump (Flux Instrument, Basel, Switzerland) and a HTS Pal autosampler (CTC analytics AG, Zwingen, Switzerland) coupled to a linear ion trap mass spectrometer LTQ-MS (ThermoFinnigan, San Jose, CA, USA), equipped with an atmospheric pressure ionisation (API) interface, Ion MAX™, operated in ESI mode. The analysis was performed on octadecyl grafted silica stationary phase Uptisphere® 120 Å ODB (150 mm × 3.0 mm, 5 μm) from Interchim (Montlucon, France). A guard column (Uptisphere® ODB, 10 mm × 4.0 mm, 5 μm) was added to the analytical column. In addition, a low dispersion in-line filter (0.5 μm frits) from Agilent (Palo Alto, CA, USA) was installed between the injector and the guard column. Elution solvents were methanol (A) and 1% (v/v) acetic acid in water (B). Mobile phase (A:B, v/v) was 50:50 from 0 to 1 min, 90:10 from 17 to 18 min and 50:50 from 19 to 20 min. The flow rate was set to 400 μL/min, and the injected volume was 10 μL.

Single runs were performed for the detection of 19-NAS. The ESI source voltage was set in negative mode and the MS instrument was operated in scan mode with the following settings for the analyses of sulfates: spray voltage = 5.0 kV; heated capillary voltage and temperature of −12 V and 300 °C, respectively; isolation width of 2 Da, normalised collision energy = 50%; activation time = 30 ms; activation *q* of 0.220 and scan time was fixed at 50 ms.

2.4. Method validation

Quantitative analysis of 19-NAS was performed using the internal standard method. A 10-point calibration curves has been calculated and fitted by a linear regression of the peak area ratios (19-NAS peak area/IS peak area) versus concentrations, using an equal weighting factor. The calibration was established over the range 0.2–20.0 ng/mL (0.2, 0.5, 1, 2, 4, 6, 8, 10, 15 and 20 ng/mL) with calibration standards spiked in a urine sample (female, Caucasian, 3 years old) free of 19-NA and 19-NE glucuronides. Data were processed using Xcalibur LCQuan software package from ThermoFinnigan.

An assay validation for the analysis of 19-NAS was performed regarding the limit of detection (LOD), lowest limit of quantification (LLOQ), intra-day and intra-assay precisions, inter-assay precision. The limit of detection (LOD) was defined as the concentration that produced a signal three times above the

noise level of a blank preparation. The lowest limit of quantification (LLOQ) for 19-NAS was experimentally chosen as the minimal concentration in urine samples leading to a deviation between measured and nominal concentration less than 20% [21,22]. For evaluation of intra-day precision, a urine sample (male, Caucasian, 25 years old) containing 19-NAS at 2 ng/mL was extracted and analysed 5 times on the same day. Five urine samples of the same individual (male, Caucasian, 25 years old) and five urine samples of five different Caucasian males (from 22 to 28 years old) were fortified with 19-NAS (2 ng/mL) and then processed on the same day to evaluate inter-assay precisions. Inter-assay accuracy and precision were determined by repeated analysis ($n = 10$) performed on 10 different days. The precision is expressed as the coefficient of variation (CV (%)), and the accuracy as the percentage of deviation between nominal and measured concentration.

The recovery of 19-NAS including ion suppression and extraction was determined at a concentration of 2 ng/mL in five urine samples (female, Caucasian, 3 years old). Recovery was calculated by comparing the absolute peak area of the analyte in the spiked urine versus the peak area of the analyte in the injection buffer.

3. Results and discussion

3.1. Chromatography and mass spectrometry

As observed in previous works, negative ESI analysis consistently produce more stable ion signal for steroid conjugates than positive ion analysis [15,20]. In MS spectra of 19-NAS and d_4-AS, the deprotonated molecule $[M-H]^-$ was the base peak in negative electrospray ionisation mode (ESI-) with no significant fragment ions in the range $m/z = 90–400$ (Fig. 2). For 19-NAS, the product ion spectra are characterised by a base peak at m/z 97 (HSO_4^-). This ion is generally observed when a sulfate group is bound to an acyclic ring of a steroid [16,23]. The MS/MS ionisation spectrum of d_4-AS displays two peaks at m/z 97 and 98 in the range of $m/z = 80–400$ (Fig. 2). Similarly to the MS/MS ionisation spectrum of NAS, the signal at m/z 97 with an intensity of about 10% correspond to the formation of the HSO_4^- fragment. The base peak at m/z 98, corresponding to a shift of 1 Da with respect to the peak at m/z 97 may be explained by the formation of the DSO_4^- product ion. This originates from the migration to the sulfate group of a deuterium at position 2 or 4 of the steroid moiety. The formation of this fragment was already

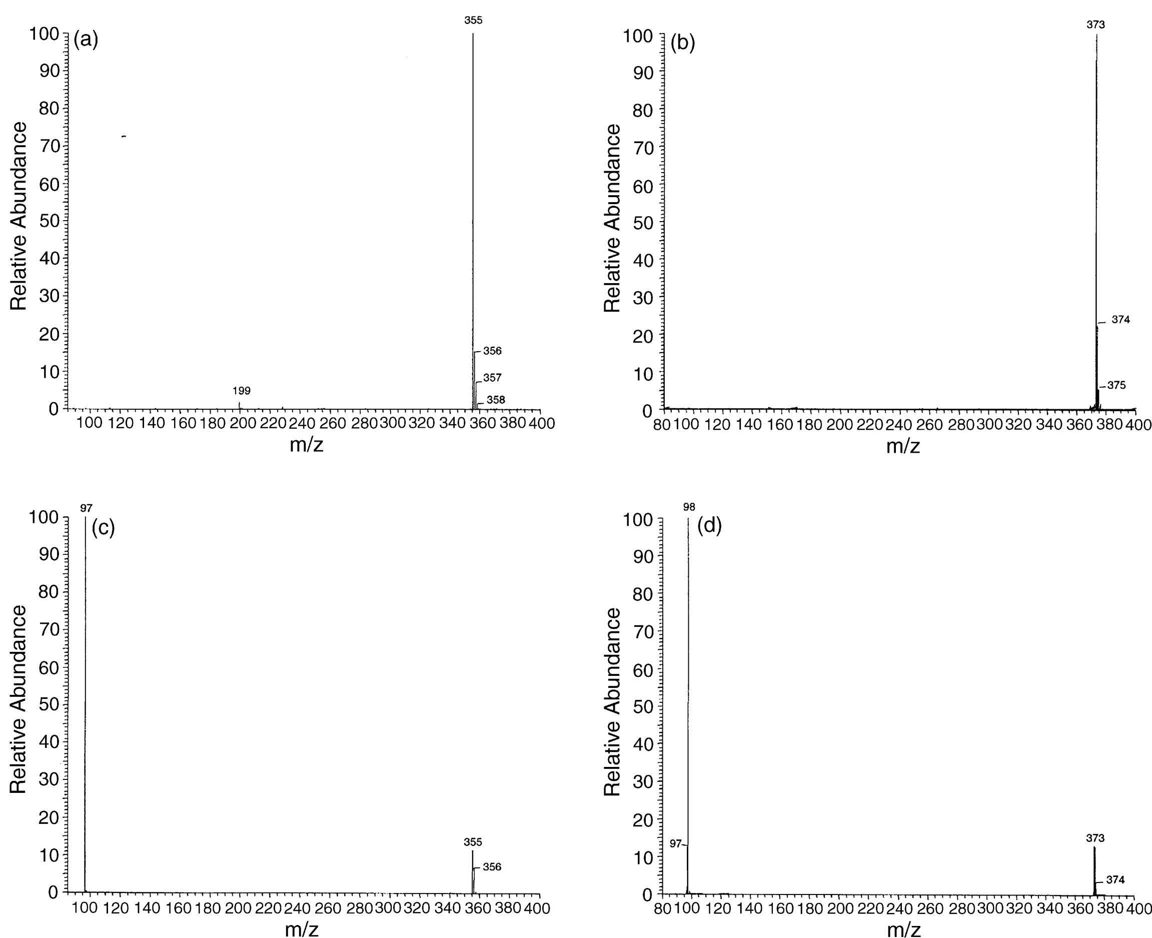

Fig. 2. ESI(-) MS spectra of 19-norandrosterone sulfate (a) and d_4-androsterone sulfate (b) showing the formation of $[M-H]^-$ ion. Panels (c and d) represent the ESI(-) MS/MS spectra of 19-norandrosterone sulfate and d_4-androsterone sulfate, respectively. Precursor ion for MS/MS experiments: NAS ($m/z = 355$), d_4-AS ($m/z = 373$). Each steroid sulfate (10 μg/mL in methanol) was infused by syringe at 20 μL/min into LC mobile phase (methanol:1% acetic acid in water, 70:30, v/v) with a flow of 100 μL/min to the mass spectrometer.

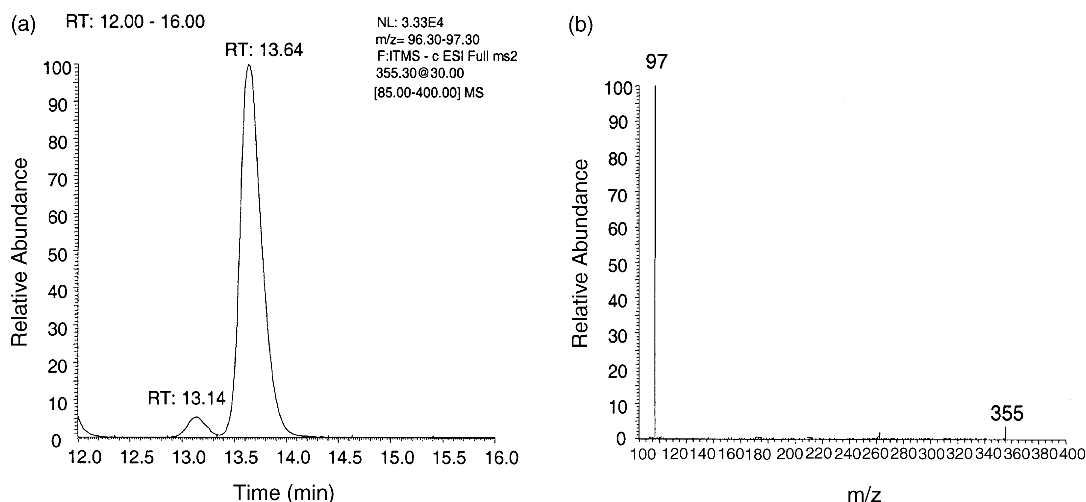

Fig. 3. Extracted ion chromatogram of 19-NAS (R_T = 13.64 min) in a positive urine sample (a) and the MS/MS spectrum (precursor ion m/z = 355) of the non-identified peak at R_T = 13.14 min (b).

observed in the MS/MS ionisation spectrum of d_3-testosterone sulfate [13].

To detect and quantify the analyte at subnanogram/mL concentration levels, we followed a protocol based on quaternary SPE for sample purification. This procedure allows the selective separation of free, glucuronide and sulfate forms of steroids in urine probes [20]. For LC-MS/MS analyses of the sulfate fraction, we used chromatographic conditions to separate the compounds of interest from other sulfate compounds of endogenous or exogenous origin. For that purpose, a series of urine samples originating from excretion studies of 19-norandrostendione [24] were analysed with the transitions at m/z 355 → m/z 97 for 19-NAS, and m/z 373 → m/z 98 for d_4-AS. Fig. 3 shows the extracted ion chromatograms with complete separation of 19-NAS and a compound of lower intensity, displaying a similar full scan MS/MS spectrum. It may be hypothesised that this compound is either 19-noretiocholanolone or 19-norepiandrosterone, two stereoisomers excreted in the urine as sulfate conjugates [10,11]. As the reference compounds were not available, it was therefore not possible to clearly identify the metabolite.

Using the chromatographic conditions described previously, it is noteworthy that the retention time obtained for 19-NAS and d_4-AS were of 13.7 min (CV 4.0%) and 15.2 min (CV 4.4%) for 100 urine assays, respectively. These results show a good reproducibility of the retention times of both compounds. Application of other elution conditions to shorten time analysis was tested. Nevertheless, it resulted in coelution of NAS with the unidentified sulfoconjugated steroid compound and the impossibility to discriminate both compounds with their MS/MS spectrum.

3.2. Assay validation

According to WADA criteria [25], identification of both 19-NAS and d_4-AS in urine sample was ensured by (1) LC chromatographic retention time agreement within 2% of the retention time of reference material analysed in the same batch and (2) measurement of full MS/MS spectrum between m/z 50 and 500 and comparison with MS/MS spectrum of standards (Fig. 2).

Calibration curves were obtained by analysis of 0.2–20 ng/mL of 19-NAS in urine samples estimated as the relative response to d_4-AS. Curves were linear with r^2 higher than 0.995. The instrument detection limit for the analyte was of 40 pg/mL. The extensive chromatography of the sample was essential to reduce ion suppression that could impair detection of 19-NAS at sub-ng/mL concentration level. As urine is considered as a complex matrix, it is fundamental to investigate the suppression or enhancement effect on the MS signal of the analytes [26]. It has been verified that matrix effect is not a reproducible or repeatable phenomenon between samples analysis and thus results in significant error in quantification procedures [27]. To assess these effects in ESI on both 19-NAS and d_4-AS, a postcolumn infusion system with the continuous infusion of analyte solution between the analytical column and the MS source was employed [26,28]. Fig. 4 represents post-column infusion chromatograms of both 19-NAS and d_4-AS obtained by LC-MS/MS (ESI) in

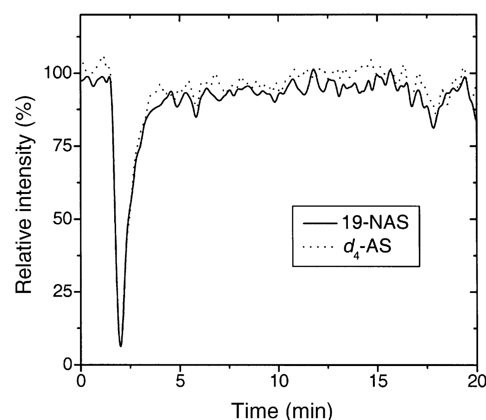

Fig. 4. LC-MS/MS ESI(-) postcolumn infusion chromatograms for 19-NAS and d_4-AS in an extracted urine sample. The analytes (1 μg/mL in methanol) were continuously infused postcolumn with a syringe pump at a flow rate of 20 μL/min.

Table 1
Assay validation parameters of 19-NAS at a nominal concentration of 2 ng/mL

Analyte recovery[a] %	Intra-day ($n=5$)	Intra-assay ($n=5$)	Inter-assay ($n=10$)	
	Precision CV (%)	Precision CV (%)	Precision CV (%)	Accuracy bias (%)
94 (12)	2.7	6.6[b], 5.8[c]	14.3	−1.0

[a] The value was the result averaged from five duplicate samples analysed on the same day. The standard deviation of the extraction recovery is given in parenthesis.
[b] Urine samples from a Caucasian male.
[c] Urine samples from five Caucasian males.

Table 2
LC-MS/MS quantification of 19-NAS in two urine samples U_1 and U_2 of athletes using (A) a standard curve and (B) the standard-addition method

	U_1	U_2
19-NA[a] [ng/mL]	35.2	32.7
(A)		
Mean 19-NAS detected [ng/mL]	8.4	19.3
CV (%), $n=3$	2.7	6.1
95% Confidence intervals	7.9–8.8	17.0–21.6
(B)		
Concentration of 19-NAS [ng/mL]	7.3	21.1
CV (%)	10.5	5.4
95% Confidence intervals	5.9–8.9	18.9–23.3

[a] Determination by GC-MS of total of free 19-NA and its glucuronide expressed as mass concentration of 19-NA in urine sample.

the negative mode. A significant ion suppression is observed within 5 min after injection for both the analyte and the deuterated internal standard. However, at the retention time of the investigated compounds (between 12 and 16 min), no significant loss of the MS/MS signal is observed. In addition, it is worth noting that the purification sample procedure using three SPE extraction steps does not affect significantly the complete 19-NAS recovery (Table 1).

Precision and accuracy were determined at a nominal concentration of 2 ng/mL of 19-NAS in urine samples. This concentration was arbitrarily chosen for the reason that an adverse analytical finding is reported when the total of free 19-NA and its glucuronide expressed as mass concentration of 19-NA in urine is greater than the threshold of 2 ng/mL established by WADA. Results obtained for intra-day and intra-assay precision, and inter-assay precision and accuracy are presented in Table 1. It is interesting to note that the intra-assay precision ($n=5$) is of about 6%, independently of the selected urine samples. Despite testing have been performed on five different urine samples, this finding shows that urine matrix does not impair the quantification of 19-NAS in urine samples. As summarised in Table 1, the inter-assay precision does not exceed 15%. Accuracy was evaluated by determining the percentage error of the mean concentration determined from analyses of 10 samples spiked at 2 ng/mL of 19-NAS. It was found that accuracy does not deviate beyond 1.0%.

Specificity of the method was tested with urine samples from 20 Caucasian individuals containing no detectable amount of 19-norandrosterone and 19-noretiocholanolone glucuronides as determined by the GC-MS procedure with a LOD of about 500 pg/mL [29]. In all urine specimens, 19-NAS was not detected and furthermore no interfering signals were observed at the retention time of the analyte.

3.3. Application to urine samples of athletes

Two urine samples from two professional athletes were selected for quantification of 19-NAS. Both U_1 and U_2 samples with a density of 1.025 and 1.016 [29], respectively, were found to contain concentration of 19-norandrosterone greater than the threshold of 2 ng/mL established by WADA (Table 2). Therefore, it was investigated if these samples from routine analysis were containing the sulfated form of 19-NA.

The identification of 19-NAS in both samples U_1 and U_2 was assessed following both criteria mentioned above for specificity. Quantitative analysis of 19-NAS was performed first using the internal standard method with 10-point calibration curves. As listed in Table 2, it was found that the mean concentration of 19-NAS was of 8.4 and 19.3 ng/mL in urine samples U_1 and U_2, respectively. The quantification was repeated in three different batches and showed a precision less than 7%. For both samples, the analyte concentration was comprised in the concentration range of the calibrators.

To validate these results and test whenever the matrix of the samples could change the analytical sensitivity of the method, quantification of 19-NAS in both samples U_1 and U_2 was also performed using the standard addition. To remain in the linear range of the MS/MS analyte response, a four-fold dilution of the samples and subsequent addition of 2, 4, 6, 8, 10 and 12 ng/mL of 19-NAS was performed. Using Matlab software (Version 6.1.0) with Statistics Toolbox (Version 3.0), linear regression of the analyte peak area versus added 19-NAS standard amounts allowed the determination of 19-NAS concentration in urine samples U_1 and U_2 together with 95% confidence intervals (Table 2). Using this method, the calculated concentrations of 19-NAS were of 7.3 and 21.1 ng/mL in urine samples U_1 and U_2, respectively (Table 2). For the standard addition method, the regression coefficient r^2 were higher than 0.996 and the slopes of the linear function were similar to those obtained for the calibration curves (0.6926 versus 0.6952).

It is noteworthy that both the standard addition and internal standard methods provided calculated concentrations of 19-NAS with differences of 14 and 9% for urine samples U_1 and U_2, respectively. In addition, the overlap of the 95% confidence intervals determined from both quantification methods is significant for samples U_1 and U_2. Based on these results, it appears that this LC-MS/MS method for direct quantification of 19-NAS in urine samples does not depend significantly on urine matrix. Conversely, the same experimental settings for quantification of the glucuronide form of 19-norandrosterone (19-NAG) in urine sample, using the corresponding deuterated internal standard (d_4-19-NAG), did not yield satisfying results in terms intra-assay precision for a nominal concentration of 10 ng/mL (data not shown). Although the extraction method used in this work

allows selective separation of the free, glucuronide and sulfate forms of steroids [20], subsequent improvement of the purification method have to be undertaken, in order to reach the required analytical performances for anti-doping analyses of glucuronide species.

4. Conclusion

A sensitive LC-MS/MS method for direct detection and quantification of 19-norandrosterone sulfate in human urine was developed. The proposed method does not require chemical/enzymatic hydrolysis, or pre-purification with HPLC, and proved to be satisfactory with respect to accuracy and reproducibility at nominal concentration of 2 ng/mL.

For quantification of 19-NAS in urine samples of athletes, the use of two different quantitation procedures (calibration curves and standard addition) yielded similar results. It demonstrated the presence of 19-NAS at concentration higher than 5 ng/mL in two urine samples containing about 30 ng/mL of 19-NA (free 19-NA + 19-NAG). Therefore, this assay may be applied for the study of phase II metabolism of sulfate 19-norsteroids and as a complementary method to detect misuse of nandrolone or nandrolone precursors.

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