Detection of stanozolol *O*- and *N*-sulfate metabolites and their evaluation as additional markers in doping control

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Abstract

Stanozolol (STAN) is one of the most frequently detected anabolic androgenic steroids in sports drug testing. STAN misuse is commonly detected by monitoring metabolites excreted conjugated with glucuronic acid after enzymatic hydrolysis or using direct detection by liquid chromatography tandem mass spectrometry (LC-MS/MS). It is well known that some of the previously described metabolites are the result of the formation of sulfate conjugates in C17, which are converted to their 17-epimers in urine. Therefore, sulfation is an important phase II metabolic pathway of STAN that has not been comprehensively studied. The aim of this work was to evaluate the sulfate fraction of STAN metabolism by LC-MS/MS to establish potential long-term metabolites valuable for doping control purposes. STAN was administered to six healthy male volunteers involving oral or intramuscular administration and urine samples were collected up to 31 days after administration. Sulfation of the phase I metabolites commercially available as standards was performed in order to obtain MS data useful to develop analytical strategies (neutral loss scan, precursor ion scan and selected reaction monitoring acquisitions modes) to detect potential sulfate metabolites. Eleven sulfate metabolites (M-I to M-XI) were detected and characterized by LC-MS/MS. This paper provides valuable data on the ionization and fragmentation of *O*-sulfates and *N*-sulfates. For STAN, results showed that sulfates do not improve the retrospectivity of the detection compared to the previously described long-term metabolite (epistanozolol-*N*-glucuronide). However, sulfate metabolites could be additional markers for the detection of STAN misuse.

Keywords: Stanozolol, sulfate metabolites, LC-MS/MS, anabolic steroids, doping analysis

1 Introduction

The anabolic androgenic steroids (AAS) are a class of performance enhancing substances prohibited in sports by the World Anti-doping Agency (WADA) at all times.

^[1] AAS are in most cases extensively modified by phase-I and phase-II metabolism and excreted in urine mainly as glucuronide and sulfates. Due to their long-lasting beneficial effects, utmost retrospectivity needs to be achieved by targeting the most long-term metabolite of each of the AAS. In the last years, sulfate metabolites have been described for several AAS. In most cases, they helped expand the detection time.^[2-7]

Among the AAS, stanozolol (STAN, 17 β -hydroxy-17 α -methyl-5 α -androst-2-eno(3,2-c)-pyrazole, Figure 1, compound **1**) is one of the most frequently detected AAS in doping control analyses.^[8] Apart from the common structure to all AAS, STAN contains a pyrazole ring attached to the steroidal A-ring which provides some analytical and metabolic peculiarities.

A large number of STAN metabolic studies have been performed during the years with the aim of expanding the window of opportunity for detecting its misuse.^[9-13] The first metabolic products described were the monohydroxylated derivatives 3'-hydroxy-stanozolol (3STAN), 4 β -hydroxy-stanozolol (4STAN) and 16 β -hydroxy-stanozolol (16STAN) (Figure 1, **2-4**) as well as corresponding analogues epimerized in C-17 (Figure 1, **5**).^[10,11] Although presenting some difficulties in its detection, 3STAN was initially the target analyte, and it is still used in most anti-doping laboratories for the long-term detection when using the conventionally employed gas-chromatography-mass spectrometry (GC-MS(/MS)) methods.^[10,14,15] GC-MS(/MS) has been the technology traditionally used for the detection of steroids but it presents some limitations due to the

need of derivatization, especially challenging for steroid metabolites with a highly conjugated system or containing a large number of hydroxyl groups.^[16] The detection of this metabolite was latter achieved using liquid chromatography-tandem mass spectrometry (LC-MS/MS).^[17] This technique presented several advantages such as the reduction of sample pretreatment and the possibility of detecting the other hydroxylated metabolites, 4STAN and 16STAN (with poor GC-MS qualities). These metabolites showed considerable greater concentrations in some athlete's samples.^[17]

Besides the benefits aforementioned, the implementation of LC-MS/MS technology represented an excellent alternative that provided a gain in sensitivity resulting in continuously decreasing limits of detection (LODs) and the possibility to directly detect phase II metabolites.^[17-19] The utility of direct analysis of glucuronides together with the commercialization of 3'-hydroxy-stanozolol glucuronide (3STAN-G) (Figure 1, **6**) allowed the development of a method that permitted limits of detection of 25-50 pg/mL.^[20] The phase II metabolites of 4STAN and 16STAN are still not completely characterized (position of the glucuronide moiety is not known) nor commercially available (Figure 1, **7-8**). Thereafter, two glucuronide metabolites resistant to hydrolysis, epi-stanozolol-1'*N*-glucuronide (eSTAN-*N*-G) (Figure 1, **9**) and stanozolol-1'*N*-glucuronide (STAN-*N*-G) (Figure 1, **10**), were directly detected and eSTAN-*N*-G enabled the determination of drug abuse for up to 28 days after oral administration.^[21] Characterization of these metabolites has been recently achieved by ion mobility and nuclear magnetic resonance (NMR) spectroscopy.^[22] Up to now, studies have focused in glucuronoconjugated metabolites obtained using both, hydrolysis with β -glucuronidase enzymes and direct detection. Sulfate metabolites have not been previously studied. However, the detection of eSTAN-*N*-G gave evidence that the formation of

diconjugates (glucuronide/sulfate) was taking place. Formation of the epimers in C17 is due to the formation of a sulfate. Sulfation at the 17 β -hydroxy of a 17 β -hydroxy-17 α -methyl steroid is sterically influenced and spontaneously decomposes in urine to yield several degradation products, being the epimer the most abundant one.^[23,24]

The aim of the present study was to identify metabolites of STAN conjugated with sulfate using LC-MS/MS analysis. For this purpose, STAN sulfate metabolites were synthesized to study the MS behavior. With this information and previous knowledge about sulfates, several methods were developed and applied to excretion study urines for the identification and characterization of new sulfate metabolites. Finally, their potential for the improvement of the detection of STAN misuse was evaluated in comparison with previously described metabolites.

2 Experimental

2.1 Chemicals and reagents

STAN was obtained from Sigma (Steinheim, Germany). 3STAN, 4STAN, 16STAN and 3STAN-G 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, were used as internal standards (IS).

Acetonitrile (ACN) (LC gradient grade), methanol (MeOH) (LC grade), formic acid (LC/MS grade), ammonium formate (LC/MS grade), sodium hydroxide, sodium

dihydrogen phosphate and aqueous ammonia solution were obtained from Merck (Darmstadt, Germany). Di-sodium hydrogen phosphate was supplied by VWR Chemicals (Leuven, Belgium). N,N-Dimethylformamide (DMF), 1,4-dioxane, sulfur trioxide pyridine complex were obtained from Sigma-Aldrich. Sep-Pak Vac RC C18 (500 mg) and Oasis WAX (60 mg) cartridges were purchased from Waters (Milford, Massachusetts, USA). Milli-Q water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.2 Synthesis of sulfate metabolites

A previously reported procedure for the synthesis of sulfate steroids ^[25] was applied to STAN and its main phase I metabolites (3STAN, 4STAN and 16STAN). 1 mg of starting material was weighted on 2 mL screw glass vials. 100 µL of 1,4-dioxane and 100 µL of a fresh solution of sulfur trioxide (100 mg/mL) in dry DMF were added in the vial. The vial was closed immediately and the reaction was carried on at room temperature for 4 hours. The reaction was then quenched with water (1.5 mL), and passed through an Oasis WAX SPE cartridge previously conditioned with MeOH (1 mL) and water (3 mL). The column was then washed with NaOH 0.1 M (3 mL), 0.05 M phosphate buffer pH 7 (3 mL) and finally with water (3 mL). Free steroids (non-reacted starting materials) were eluted with MeOH (3 mL). Sulfates were eluted with NH₃ 5% in MeOH. Samples were evaporated to dryness under nitrogen stream in a bath at 40°C. The dry extract was reconstituted with 200 µL of a mixture of deionised water:ACN (9:1, v|v) and 10 µL were analyzed by LC-MS/MS.

2.3 Excretion study samples

Clinical studies consisted of the administration of a single oral dose of STAN (6 mg) (4 volunteers) or a single intramuscular injection (IM) (50 mg) (2 volunteers). Urine

samples were collected at baseline and from 0-4h, 4-8h, 8-12, 12-24h, 24-36h, 36-48h, 48-72h, 72-96h, 96-108h, 108-120h and daily spot morning urine samples from day 6 to day 31 after administration.

Ethical approval was granted by Ethical Committee of our Institute (Consorti 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.

Blank urine samples were collected from ten healthy volunteers and used to check the specificity of the method. Positive doping test samples for STAN with authorization to be used for research purposes were also analyzed. All urine samples were kept frozen at $< -20^{\circ}\text{C}$ until analysis.

2.4 Sample preparation

Extraction of phase II conjugates was based on a previously described method with minor modifications.^[26] Briefly, after the addition of 20 μL of the IS solution (MET, d3-NAN-S and d3-T-G at 1 $\mu\text{g/mL}$, and d4-And-G at 5 $\mu\text{g/mL}$), urine samples (2,5 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 $^{\circ}\text{C}$. The extracts were 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.

In order to obtain more concentrated extracts for the detection of new metabolites, two aliquots of 2.5 mL were extracted and combined before evaporation of the MeOH.

2.5 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 20 L/min, and the cone gas flow was 50 L/h. The nitrogen desolvation temperature was 450 °C, and the source temperature was 120 °C. Capillary voltages were set at 3.5 and 2.5 kV in positive and negative ionization modes, respectively.

Chromatographic separations were carried out on an Acquity UPLC® system (Waters Corporation, Milford, MA, USA) 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. Mobile phase composition was 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). The following gradient program was used to detect potential sulfate metabolites: 0 min, 20 % B; 2 min, 20% B; 15 min, 40% B; 16 min, 70% B; 17 min, 95% B; 18 min, 95% B; 18.5 min, 20% B; 20 min, 20% B. Only for the evaluation of the synthesized material, a different mobile phase composition and gradient program were used. Solvent A consisted of 0.01% formic acid and 1.0 mM ammonium formate in water and solvent B was 0.01% formic acid and 1.0 mM ammonium formate in MeOH. The gradient program was as follows: 0 min, 5 % B; 0.5 min, 5% B; 5 min, 95% B; 5.2 min, 95% B; 5.3 min, 5% B; 6.5 min, 5% B.

3 Results and discussion

3.1 Synthesis and mass spectrometric behavior of STAN sulfate metabolites

To study the mass spectrometric behavior of STAN sulfate metabolites, sulfation of the parent compound (STAN) and phase I metabolites commercially available as standards (3STAN, 4STAN and 16STAN) was performed (Figure 1, **1-4**). Each of the compounds has multiple potential sites of sulfation and depending on where the sulfate gets conjugated, a different ionization and collision induced dissociation (CID) behavior is expected.^[27] After the synthesis procedure, only one sulfate metabolite per each compound was detected by LC-MS/MS analysis. Sulfates of STAN, 3STAN and 4STAN could be detected immediately after the reconstitution in mobile phase. However, the re-injection of the same vials after 24 hours demonstrated the degradation of these products.. Only one of the synthesized sulfates, 16 β -hydroxy-stanozolol 16 β -sulfate (16STAN-S), was a stable product. The obtained data are discussed in the following sections.

3.1.1. *Unstable products*

Stanozolol 17-sulfate (STAN-S) and 3'-hydroxy-stanozolol 17-sulfate (3STAN-S) were detected immediately after the synthesis procedure (Figure S-1, supporting material (supp. mat.)). After 24 hours, only the peaks corresponding to the parent compounds (RT: 5.37 and 4.94 min) and peaks assumed to be the 17-epimers (RT: 5.62 and 5.27 min) for STAN and 3STAN, respectively, were detected (Figure S-1, supp. mat.). These results are in agreement with the well-known instability of 17 α -methyl-17 β -sulfates in aqueous solution and suggest sulfation in C17 position and subsequent degradation of the sulfate conjugates.^[23,24] For 4 β -hydroxy-stanozolol sulfate (4STAN-S) only one chromatographic peak at the retention time of the parent compound (RT: 4.92 min) was

detected after degradation of the sulfate (Figure S-1, supp. mat.). These metabolites are probably produced in the human body but their inclusion to doping control procedures is not recommended due to their hydrolysis in urine.

The CID spectra in positive and negative ionization modes were evaluated. In positive ionization mode, the NL of 80 Da (loss of a sulfur trioxide group), common to sulfate conjugates, with or without a water molecule (NL of 98 Da) was detected for the three synthesized sulfates. Subsequently, product ions corresponding to the free steroid were observed.^[12] In negative ionization mode, they all yielded the characteristic product ion at m/z 97, corresponding to the hydrogensulfate anion. As an example, product ion mass spectra of STAN-S in positive and negative ionization modes (at low and high collision energies (CE)) are shown in Figure 2A.

3. 1. 2. *Stable product*

The synthesis procedure for 16STAN produced one stable product. The analysis of the same vial after 24 h, 1 month and 2 years showed the same results (Figure S-1, supp. mat.). Therefore, it could not be conjugated in C17 because same behavior as the unstable products would have been observed. Furthermore, the conjugation in $-N$ is improbable for two main reasons. First, the formation of N -sulfates seems to not be favorable under these synthesis conditions; otherwise, N -sulfates would have been synthesized for the other metabolites. Second, the hydroxyl group in C16 has major reactivity and, therefore, 16 β -hydroxy-stanozolol 16 β -sulfate (16STAN-S) was the expected product of the sulfation reaction.

In positive ionization mode, the synthesized product ionized as $[M+H]^+$ through the nitrogen atom in the pirazol ring. In negative mode, it exhibited an abundant $[M-H]^-$ ion (Figure 2, B). The CID was also evaluated. In positive mode, the neutral loss (NL) of 80

Da was observed. Subsequently, after the loss of the sulfate group, CID fragmentation was the same as for the free steroid and showed the ion at m/z 81, 91 and 105 specific of STAN metabolites without a modification in the pirazol or A-ring.^[17] In negative ionization mode, only one abundant product ion at m/z 97 was observed when using both, low and high CE (Figure 2, B).

The observed mass spectrometric behavior was in agreement with the proposed structure. Moreover, the stability of the compound was an additional confirmation that sulfation took place in the hydroxyl group in C16 position instead of the hydroxyl in C17 that would have given the corresponding epimer.

3.2 LC-MS/MS strategies for the detection of STAN metabolites excreted as sulfate conjugates

Based on the information obtained from the product ion mass spectra of the synthesized STAN sulfate metabolites as well as previously reported data^[19,27,28], different LC-MS/MS strategies were developed to directly detect sulfate metabolites; open scan methods and a selected reaction monitoring method (SRM) (Table 1). Only peaks detected in post-administration samples and not present in pre-administration samples were considered as potential sulfate metabolites of STAN.

3.2.1. Open scan methods

First of all, five precursor ion (PI) and three NL scan methods were designed (Table 1, methods 1-4). In positive mode, STAN metabolites without a modification in N- or A-rings exhibit the product ion m/z 81 (Figure 2) whereas the metabolites hydroxylated in the pirazole ring and 4-hydroxy-stanozolol metabolites yield the ions at m/z 97 and m/z 145, respectively.^[12] For this reason, PI methods of these typical product ions (Table 1, method 1) were applied. In negative mode, PI methods of m/z 97 and 80, characteristic

ions of sulfate metabolites ^[2,27,28], were also used (Table 1, method 2). The most common fragments of steroid sulfates in positive mode are the NLs of 80 Da and 98 Da (Figure 2, B and A, respectively). Hence, NL methods of 80 and 98 Da were also employed (Table 1, method 3). Finally, a NL of 80 Da in negative mode has also been reported for aryl *O*-sulfates and *N*-sulfates.^[27] Therefore, a NL method of 80 Da in ESI negative mode was also developed (Table 1, method 4).

3.2.2. *Select reaction monitoring methods*

At the same time, a SRM method was developed (Table 1, method 5). This strategy has been reported to be more sensitive than untargeted methods.^[29] To calculate the theoretical transitions, different metabolic pathways were considered (Figure S-2, supp. mat.). The list of putative sulfates metabolites covered all the phase I metabolites previously described.^[10,12] In positive mode, transitions from $[M+H]^+$ of all potential sulfate metabolites to m/z 81, 97 and 145, characteristic ions at high CE, were set up. For STAN-S, a transition to the product ion m/z 311 was introduced because it was seen in the mass spectra of synthesized compound (Figure 2, A). These transitions were not applied to potential reduced metabolites because the only possible reduction reactions would change the pyrazol ring. In negative ionization mode, theoretical transitions from $[M-H]^-$ to m/z 80, m/z 97 and $[M-H-80]^-$ were used. In summary, 55 ion transitions corresponding to 12 MMs (representing many putative sulfate metabolites) were included into the method (Table 1, method 5 and Figure S-2 (sup. mat.)).

3.3 Detection of STAN metabolites excreted as sulfates

The methods were applied to samples collected before and after STAN administration (oral and IM). After combining information of all these methods, eleven sulfate

metabolites (M-I to M-XI) were detected in post-administration samples. No peaks at the same retention time (RT) were observed in pre-administration samples.

The MMs, RTs and product ions obtained in positive and/or negative ionization modes are listed in Table 2. LC-MS/MS chromatograms of a pre-administration sample and a post-administration sample are included in Figure S-3 (supp. mat.).

3.4 Characterization of STAN sulfate metabolites

3.4.1. *The structures of the detected sulfate metabolites have been proposed after evaluation of the data obtained from the product ion mass spectra acquired in positive and negative modes (Table 2, Figure 3), previous knowledge about sulfates and data acquired from the synthesized products. Metabolites M-I, M-II and M-III*

M-I, M-II and M-III had the same MM (424 Da), indicating one hydroxylation, and they all ionized in both ionization modes (Table 2). Moreover, they all shared the same product ions in the positive CID spectra (m/z 345, 327, 105, 95 and 81). In negative mode, they yielded only one product ion (m/z 97) (Table 2). Therefore, these three compounds are most probably stereoisomers.

The RT (4.0 min) and CID spectra in positive and negative modes obtained for M-I (Figure 2, C) matched perfectly with the data obtained for 16STAN-S after the synthesis procedure (Figure 2, B). These results confirmed that M-I was 16STAN-S with the sulfate moiety in the hydroxyl group in C16 position. Therefore, for metabolites M-II and M-III, the possible candidates are the sulfate conjugates of 16 α -hydroxy-stanozolol and 16 α -hydroxy-17-epistanozol that have been previously reported as STAN metabolites.^[10] However, the sulfate conjugate of 16 β -hydroxy-17-epistanozol cannot be discarded. Metabolite M-II was not found in any sample of the excretion studies but

it was detected in positive doping test samples where the other STAN metabolites were detected in very high abundances in comparison to the excretion study urines.

3. 4. 2. Metabolites M-IV and M-V

The MM of these metabolites (408 Da) suggested sulfation of STAN. These compounds were only detected under negative ionization (Table 2). Thus, the sulfate moiety could only be conjugated to the pyrazol ring due to the instability of sulfate conjugates linked to a 17 β -hydroxy group of STAN.

The two nitrogen atoms of the pyrazol ring are potential sites of sulfation. The mass spectra of the detected metabolites in this present work suggested that when the sulfate is conjugated through the nitrogen atom, the pyrazol ring cannot ionize in positive ionization mode (as observed for metabolites M-IV and M-V) and, in negative ionization mode the compound can fragment to several product ions, such as a NL of 80Da (SO₃) and the formation of the ion m/z 93 (Table 2, Figure 3). Figure 4 shows a scheme to explain the formation of the ion at m/z 93 under negative ionization. This interpretation together with the fact that product ion m/z 97 was not present confirmed that one of the nitrogen atoms in the pyrazole ring is conjugated to the sulfate moiety.

Figure 3 shows the mass spectra obtained for M-IV in negative mode at low and highCE. Metabolite M-V was only detected in SRM mode (407>327, 407>93). Considering that there are two nitrogen atoms that can be conjugated, two different structures are possible for M-IV and M-V. In this work, for plotting purposes, *N*-sulfate metabolites were drawn with the sulfate in position 1'. Moreover, epimerization of C17

is also possible. NMR or crystallographic studies would allow for the complete characterization of the metabolites.

3. 4. 3. Metabolites M-VI, M-VII and M-VIII

These metabolites have the same MM (424 Da) as M-I, M-II and M-III indicating one hydroxylation. However they were not detected in positive ionization mode. In negative mode, their mass spectra showed the ions m/z 343, 325, 311, 293 and 93 (Table 2). These data suggested hydroxy-stanozolol *N*-sulfates due to the existence of the NLs of SO_3 (m/z 343) and SO_3 plus water (m/z 325) and also, the presence of the ion at m/z 93 (Figure 4) by analogy to previously described metabolites (M-IV and M-V). As an example, Figure 3 shows product ion mass spectrum of $[\text{M-H}]^-$ of M-VI in ESI negative mode. Unfortunately, this was limited information to characterize these metabolites and, thus, no information of the hydroxylation sites could be obtained.

Nevertheless, considering STAN metabolism, the most feasible configurations are 16β and 16α (hydroxylation in C4 would lead to other product ions, see section 3.4.5). Epimerization of carbon atoms in C17 and the two different possibilities of *N*-sulfation should also be considered.

3. 4. 4. Metabolite M-IX

The MM of this compound (422 Da) suggested hydroxylation and oxidation reactions as phase I metabolism before conjugation with the sulfate moiety (Figure S-2, supp. mat.). This compound was not detected in positive ionization mode. In negative ionization mode, the mass spectra yielded the ions m/z 341, 311, 255 and 93 (Table 2, Figure 3) suggesting a *N*-sulfate. Considering previously reported metabolites^[10,12,21] and also the

absence of NL of water after the NL of the sulfate group, the most feasible structure for M-IX is 16-oxo-stanozolol-*N*-sulfate.

3. 4. 5. Metabolite M-X

The MM of this compound (438 Da) suggested one oxidation and two hydroxylations reactions as phase I metabolism prior the conjugation with the sulfate moiety (Figure S-2, supp. mat.). As for some of the other metabolites, this compound was not detected in positive ionization and mass spectra under negative ionization suggested an *N*-sulfate. Product ions at m/z 357 and 339 corresponded to NLs of SO_3 and SO_3 plus water. In this case ion at m/z 93 was not detected (Figure 3). This could indicate than one of the hydroxyl groups could be located near the pyrazol ring blocking the retro-Diels-Alder reaction. Considering STAN metabolism, the most feasible position for the hydroxyl group is 4, where after the NL of water a conjugated double bond will be generated. This is also in agreement with the NL of 16Da (ion at m/z 323), because the loss of methyl group in C10 will lead to double saturated ring (Figure 5). Thus, the most feasible structure for M-X is 4 β -hydroxy-16-oxo-stanozolol-*N*-sulfate. This metabolite has been previously described as unconjugated.^[12] After the NL of the sulfate moiety in the present study and the NL of the acetic acid from the $[\text{M}+\text{CH}_3\text{COO}]^-$ adduct in the previous work,^[12] same product ions (m/z 357, 339 and 323) were observed (Figure 3).

3. 4. 6. Metabolite M-XI

The MM of this compound (440 Da) suggested two hydroxylation reactions as phase I metabolism followed by the sulfate conjugation. This compound was only detected under SRM mode (439 $\rightarrow$ 359), so not much information, besides that sulfate is conjugated by the nitrogen atom to the pyrazol ring could be obtained.

For most of the detected metabolites in this work, chemical structures are only proposed. For the complete characterization of these metabolites, synthesis of the reference materials and, subsequent characterization by NMR or crystallographic studies would be required. Unfortunately, these experiments require amounts in the *mg* range and the low concentration of these metabolites in urine (in the range of ng/mL) and also, the need of a purification step makes it impossible to perform the experiments of the compounds isolated from urine.

3.5 Evaluation of excretion profiles of sulfate metabolites by LC-MS/MS

In order to evaluate the detection windows of the previously detected sulfate metabolites, an SRM method was optimized (Table 1, method 6). The ion transitions to monitor each sulfate metabolite were selected based on signal intensity and selectivity. Excretion study samples were analyzed to optimize the ion transitions for each metabolite. Cone voltage and CE were optimized to obtain maximum signal for each transition. For the final SRM method, the two ion transitions that gave the highest signal-to-noise- ratios were selected. Precursor ions, product ions and analytical parameters are listed in Table 1, method 6. Selectivity was studied by checking any interfering substance at the RT of the sulfate metabolites in 10 different blank urine samples obtained from different healthy volunteers.

This method was applied to pre- and post-administration samples from six different individuals (four after oral administration and two after IM injection). Detection times of all sulfate metabolites after oral administration are depicted in Figure 6. Metabolites could be detected from the first hours and up to 10 days after administration. Metabolites M-IV, M-IX and M-X showed the highest retrospectivity among all

sulfates. However while similar detection windows were observed for M-X for all four volunteers (8-9 days), the detection windows for M-IV and M-IX shifted between 4 and 10 days and 5 and 8 days, respectively, demonstrating considerable inter-individual variability.

Regarding IM administration, all metabolites were detected from day 1 and up to day 31 which was the last collected sample in both studies. This is probably due to a higher dose administered (50 mg) compared to the oral administration studies (6 mg) and a different adsorption into the circulation with the IM administration.

3.6 Comparison with intact glucuronide metabolites detected by LC-MS/MS analysis

For comparison purposes, the excretion study samples were also analyzed by using a recently developed method for the direct detection of phase II metabolites.^[26] This method includes five STAN glucuronides (3STAN-G (9.22 min), 16STAN-G (4.64 min), 4STAN-G (7.08 min), STAN-N-G (7.55 min) and eSTAN-N-G (10.90 min)). The SRM transitions for these metabolites are included in Table 1 (method 7) and results are shown in Figure 6. The longest detection times after oral administration were obtained for eSTAN-N-G that was detected in all four excretion studies up to 19, 21, 22 and 23 days for volunteer 1, 2, 3 and 4, respectively. These results are in accordance with the previous data that suggested eSTAN-N-G as the most long-term metabolite after a single oral dose of 5 mg in two volunteers.^[21] 3STAN-G showed a detection window between 9 and 14 days depending on the volunteer (Figure 6). For the IM samples, all metabolites were detected in all samples (from day 1 to day 31) from the two volunteers.

In summary, STAN sulfate metabolites do not increase the detection window compared to the classical glucuronide, 3STAN-G, or the previously described long-term glucuronide eSTAN-*N*-G.^[21] However, this work contributes to provide additional data of the excretion profiles of STAN metabolites for several individuals. Taking into consideration the large inter-individual and inter-ethnic variations that exist for some AAS^[30], this is very valuable information for the doping control field.

4 Conclusions

In this study, direct detection of STAN sulfate metabolite was achieved using LC-MS/MS analyses in positive and negative ionization modes. Eleven new STAN sulfate metabolites have been described. Moreover, new information about the ionization and MS/MS behavior of *N*-sulfates metabolites has been discussed and could be useful to study other steroids with similar chemical structure (e.g. prostanolol). Regarding retrospectivity, three STAN sulfate metabolites (M-IV, M-IX and M-X) were considered important. Compared to eSTAN-*N*-G, these metabolites do not improve the retrospectivity of the administration of the drug. However, the results of the present study demonstrate that sulfation plays an important role in the metabolism of STAN. M-IV was detected up to 10 days, similarly to 3STAN-G and, therefore, could be an alternative marker of STAN abuse depending on the employed analytical strategy. Finally, these sulfate metabolites could also be useful markers in other fields such as horse doping analysis or cattle feeding where STAN is also misused.

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Table 1. Mass spectrometric methods used during this study. Parameters: neutral loss (NL), precursor ion (PI), selected reaction monitoring (SRM), product ion (DI), cone voltage (CV) and collision energy (CE).

UNTARGETED DETECTION							
Method	Scan mode	Ionization mode	PI (m/z)	CV (V)	CE (eV)	Range (m/z)	
1	PI	ESI +	81	30	55	350-500	
			97		55		
			145		45		
2	PI	ESI -	97	60	35	350-500	
			80				
Method	Scan mode	Ionization mode	NL (Da)	CV (V)	CE (eV)	Range (m/z)	
3	NL	ESI +	80 98	30	10	350-500	
4	NL	ESI -	80	60	20	350-500	
Method	Scan mode	Metabolite	PI (m/z)	DI (m/z)	CV (V)	CE (eV)	
5	SRM	Putative sulfate metabolite	[M+H] ⁺	81	20	55	
			[M+H] ⁺	97	20	55	
			[M+H] ⁺	145	20	45	
			[M-H] ⁻	80	60	35	
			[M-H] ⁻	97	60	35	
			[M-H] ⁻	[M-H-80] ⁻	60	35	
TARGETED DETECTION							
Method	Scan mode	Metabolite	ESI	PI (m/z)	DI (m/z)	CV (V)	CE (eV)
6	SRM	M-I, MII, M-III	pos	425	345	25	20
			neg	423	97	60	45
		M-IV, M-V	neg	407	327	50	40
			neg	407	93	50	65
		M-VI, M-VII, M-VIII	neg	423	343	60	45
			neg	423	325	60	55
		M-IX	neg	421	341	60	40
			neg	421	255	60	52
		M-X	neg	437	357	60	35
			neg	437	339	60	40
		M-XI	neg	439	359	55	35
		Method	Scan mode	Metabolite	ESI	PI (m/z)	DI (m/z)
7	SRM	3STAN-G	pos	345	97	60	45
			neg	519	343	45	30
		16STAN-G	pos	521	81	25	65
			pos	521	345	25	40
		4STAN-G	pos	521	309	25	25
			neg	519	193	45	25
		STAN- <i>N</i> -G	pos	505	329	25	45
			pos	505	81	25	65
		eSTAN- <i>N</i> -G	pos	505	329	25	45
			pos	505	81	25	65

Table 2. STAN sulfate metabolites detected in human urine. Parameters: molecular mass (MM), retention time (RT), precursor ion (PI), product ion (DI).

Metabolite	MM (Da)	RT (min)	ESI +		ESI -	
			PI (m/z)	DI (m/z)	PI (m/z)	DI (m/z)
M-I	424	4.0	425	345, 327, 105, 95, 81	423	97
M-II*	424	2.0	425	345, 327, 105, 95, 81	423	97
M-III	424	3.4	425	345, 327, 107, 95, 81	423	97
M-IV	408	9.9	-	-	407	327, 309, 93
M-V	408	9.6	-	-	407	327, 93
M-VI	424	1.8	-	-	423	343, 325, 311, 293, 93
M-VII	424	2.3	-	-	423	343, 325, 311, 293, 93
M-VIII	424	3.8	-	-	423	343, 325, 307, 93
M-IX	422	4.6	-	-	421	341, 311, 255, 93
M-X	438	4.0	-	-	437	357, 339, 323
M-XI	440	4.4	-	-	439	359

* This compound was not detected in excretion study samples, but it was detected in some athletes' samples.

FIGURE LEGENDS

Figure 1. Brief scheme of the metabolism of stanozolol involving phase I and phase II metabolism based on literature; **1:** stanozolol (STAN), **2:** 3'-hydroxy-stanozolol (3STAN), **3:** 4 β -hydroxy-stanozolol (4STAN), **4:** 16 β -hydroxy-stanozolol (16STAN), **5:** epistanozolol (eSTAN), **6:** 3'-hydroxy-stanozolol glucuronide (3STAN-G), **7:** 4 β -hydroxy-stanozolol glucuronide (4STAN-G), **8:** 16 β -hydroxy-stanozolol glucuronide (16STAN-G), **9:** epistanozolol-1'*N*-glucuronide (eSTAN-*N*-G) and **10:** stanozolol-1'*N*-glucuronide (STAN-*N*-G).

Figure 2. Product ion mass spectra of $[M+H]^+$ and $[M-H]^-$ of two of the synthesized compounds; **A)** STAN-S (before degradation) and **B)** 16STAN-S (stable synthesized product); and **C)** metabolite M-I directly detected in urine (data was acquired at low and high CE). The proposed chemical structures are also shown.

Figure 3. Product ion mass spectra of $[M-H]^-$ of sulfate metabolites M-IV, M-VI, M-IX and M-X in ESI negative mode at low (CE= 40 eV) and high (CE= 60 eV) CE detected from positive urines. The proposed chemical structures are also shown.

Figure 4. Scheme of the possible rearrangement to explain the formation of the ion at m/z 93 detected for STAN *N*-sulfate metabolites when acquired in negative ionization mode.

Figure 5. Fragmentation scheme to explain the neutral loss of 16 Da and the absence of the ion at m/z 93 in the mass spectra of metabolite M-X in negative ionization mode.

Figure 6. Detection times of the different metabolites of STAN obtained for the four excretion studies (volunteer 1→4) after oral administration using direct LC-MS/MS analysis of sulfate (M-I to M-XI) and glucuronide metabolites (3STAN-G, 4STAN-G, 16STAN-G, STAN-*N*-G, eSTAN-*N*-G).

Ionization mode (ESI)		Proposed chemical structure	
Positive [M+H] ⁺ (CV= 20 V)	Negative [M-H] ⁻ (CV= 60 V)		
CHEMICALLY SYNTHETIZED PRODUCTS			
A	50 eV	40 eV	STAN-S
	10 eV	10 eV	
B	50 eV	40 eV	16STAN-S
	10 eV	20 eV	
SULFATE METABOLITES DIRECTLY DETECTED IN URINE			
C	50 eV	40 eV	16STAN-S
	10 eV	20 eV	

	M-IV	M-VI	M-IX	M-X
CE = 60 eV				
CE = 40 eV				
Proposed chemical structure				

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Figure S-2. SRM method developed to detect potential STAN sulfate metabolites in positive and negative ionization modes. The most common metabolic pathways (red: reduction; ox: oxidation; OH: hydroxylation) were considered. Parameters: precursor ion (PI), product ion (DI), cone voltage (CV) and collision energy (CE).....p. S-3

Figure S-3. LC-MS/MS results: SRM chromatograms (transitions corresponding to STAN sulfate metabolites) of a pre-administration sample and a post-administration sample (4-8h).....p. S-4

Figure S-1. Stability of stanozolol (STAN) sulfate metabolites. LC-MS/MS chromatograms of: A, synthesized products (sulfate metabolite); B, degradation products (after 24h); and C, reference materials of the free steroids.

For the evaluation of the synthesized material, chromatographic separations were carried out on an Acquity UPLC® system (Waters Corporation, Milford, MA, USA) 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. Mobile phase composition was 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 methanol (solvent B). The following gradient program was used to detect the synthesized sulfate metabolites: 0 min, 5 % B; 0.5 min, 5% B; 5 min, 95% B; 5.2 min, 95% B; 5.3 min, 5% B; 6.5 min, 5% B.

Figure S-2. SRM method developed to detect potential STAN sulfate metabolites in positive and negative ionization modes. The most common metabolic pathways (red: reduction; ox: oxidation; OH: hydroxylation) were considered. Parameters: precursor ion (PI), product ion (DI), cone voltage (CV) and collision energy (CE).

Metabolic pathway*	Putative sulfate MM (Da)	ESI positive				ESI negative			
		PI (m/z)	DI (m/z)	CV (V)	CE (eV)	PI (m/z)	DI (m/z)	CV (V)	CE (eV)
STAN-S + 2ox	404	405	81 97 145	20	55 55 45	-	-	-	-
STAN -S + 1ox	406	407	81 97 145	20	55 55 45	405	97 325	60	35
STAN -S	408	409	81 311	20	55 50	407	80 97 327	60	35
STAN -S + 1red	410	-	-	-	-	409	80 97 329	60	35
STAN -S + 2red	412	-	-	-	-	411	80 97 321	60	35
STAN -S + 2ox + 1OH	420	421	81 97 145	20	55 55 45	419	80 97 339	60	35
STAN -S + 1ox + 1OH	422	423	81 97 145	20	55 55 45	421	80 97 341	60	35
STAN -S + 1OH	424	425	81 97 145	20	55 55 45	423	80 97 343	60	35
STAN -S + 1red + 1OH	426	-	-	-	-	425	80 97 345	60	35
STAN -S + 2red + 1OH	428	-	-	-	-	427	80 97 347	60	35
STAN -S + 1ox + 2OH	438	439	81 97 145	20	55 55 45	437	80 97 357	60	35
STAN -S + 2OH	440	441	81 97 145	20	55 55 45	439	80 97 359	60	35

* Each MM can correspond to a metabolite resulting from the metabolic modification(s) shown in this table, or to metabolites combining an additional and equal number of reductions and oxidations.

Figure S-3. LC-MS/MS results: SRM chromatograms (transitions corresponding to STAN sulfate metabolites) of a pre-administration sample and a post-administration sample (4-8h).