Sulfate metabolites as alternative markers for the detection of 4-chlorometandienone misuse in doping control

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Abstract

Sulfate metabolites have been described as long-term metabolites for some anabolic androgenic steroids (AAS). 4-chlorometandienone (4Cl-MTD) is one of the most frequently detected AAS in sports drug testing and it is commonly detected by monitoring metabolites excreted free or conjugated with glucuronic acid. Sulfation reactions of 4Cl-MTD have not been studied. The aim of this work was to evaluate the sulfate fraction of 4Cl-MTD metabolism by liquid chromatography tandem mass spectrometry (LC-MS/MS) to establish potential long-term metabolites valuable for doping control purposes. 4Cl-MTD was administered to two healthy male volunteers and urine samples were collected up to 8 days after administration. A theoretical selected reaction monitoring (SRM) method working in negative mode was developed. Ion transitions were based on ionization and fragmentation behavior of sulfate metabolites as well as specific neutral losses (NL of 15 Da and NL of 36 Da) of compounds with related chemical structure. Six sulfate metabolites were detected after the analysis of excretion study samples. Three of the identified metabolites were characterized by LC-MS/MS and GC-MS/MS. Results showed that five out of the six identified sulfate metabolites were detected in urine up to the last collected samples from both excretion studies.

Keywords: 4-chlorometandienone, sulfate metabolites, LC-MS/MS, anabolic steroids, doping analysis, metabolism

1 Introduction

4-chlorometandienone (4Cl-MTD, 4-chloro-17 β -hydroxy,17 α -methylandrosta-1,4-dien-3-one), also known as dehydrochloromethyltestosterone or oral-turinabol (Figure 1), is a synthetic anabolic androgenic steroid (AAS) included in the list of prohibited substances of the World Anti-Doping Agency (WADA).^[1] Further, it is one of the most reported AAS in doping control laboratories.^[2]

In sports drug testing, metabolic studies of AAS are necessary to identify the metabolites that provide the utmost retrospectivity. Conventional approaches have focused on the detection of the most abundant metabolites after hydrolysis with β -glucuronidase enzymes to release the phase I metabolite and analysis by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).^[3-7] In recent years, some studies using LC-MS/MS technology for the direct detection of phase II metabolites (glucuronides and sulfates) revealed the importance of minor/long-term metabolites that remain undetected when using common methods.^[8-19] For some steroids, sulfate metabolites really improved the retrospectivity of the detection.^[9-11,15-17,19] In line with these discoveries, new screening methods based on the direct analysis of the phase II metabolites have been developed.^[20,21]

The metabolism of 4Cl-MTD has been widely investigated and the main metabolites described are presented in Figure 1. The first studies revealed the presence of the parent drug (**1**) together with three major hydroxylated metabolites: 6 β -hydroxy-4Cl-MTD (metabolite **2**), 16 β -hydroxy-4Cl-MTD (**3**) and 6 β ,16-dihydroxy-4Cl-MTD (**4**) in post-administration urine.^[22,23] Later, 6 β ,12-dihydroxy-4Cl-MTD (**5**) and minor amounts of epi4Cl-MTD (**6**) were also identified.^[24] Afterwards, a more in depth study of 4Cl-MTD metabolism in human urine revealed an important metabolite detectable in urine up to 14 days after administration that

was characterized as 4-chloro-3 α ,6 β ,17 β -trihydroxy-17 α -methyl-androst-1-en-16-one (**7**).^[25] Thereafter, the existence of 18-nor-17-hydroxymethyl metabolite of 4Cl-MTD, similar to metandienone, was demonstrated.^[5,6] This metabolite, 4-chloro-18-nor-17 β -hydroxymethyl,17 α -methylandrosta-1,4,13-trien-3-one (**8**), was found to be as valuable as metabolite **7**, both being detectable 22 days after administration by GC-MS/MS.^[26] Finally, six long-term metabolites were identified as 4-chloro-17 α -methyl-5 β -androstan-3 α ,16,17 β -triol (**9**), 4-chloro-18-nor-17 β -hydroxymethyl,17 α -methylandrosta-4,13-dien-3 α -ol (**10**) and its 17-epimer (**epi10**), 4-chloro-18-nor-17 β -hydroxymethyl,17 α -methyl-5 β -androsta-1,13-dien-3 α -ol (**11**), 4-chloro-18-nor-17 β -hydroxymethyl,17 α -methyl-5 β -androst-13-en-3 α -ol (**12**), its 17-epimer (**epi12**). Metabolite **12** and, to a lesser extent, its epimer (**epi12**) showed to be superior regarding retrospectivity to the other known 4Cl-MTD metabolites, such as metabolites **7** and **8**, in most of the cases.^[5] Up to now, studies have only focused in glucuronoconjugated metabolites obtained after hydrolysis with β -glucuronidase enzymes and the detection of the released phase I metabolites by GC-MS and GC-MS/MS. Sulfate metabolites have not been previously studied.

The goal of the present study was to evaluate the sulfate fraction of 4Cl-MTD metabolism using LC-MS/MS analysis and to assess the potential of sulfate metabolites as alternative markers for the detection of 4Cl-MTD misuse compared with previously described metabolites.

2 Materials and methods

2.1 Chemicals and reagents

6 β -hydroxy-4Cl-MTD and 17 β -boldenone sulfate were obtained from NMI (Pymble, Australia). Boldenone and methyltestosterone were obtained from Sigma (Steinheim, Germany). *Tert*-butylmethyl ether (TBME, for analysis), ethyl acetate (LC grade), acetonitrile (ACN) (LC grade), methanol (MeOH) (LC grade), formic acid (LC-MS grade), potassium carbonate, sulfuric acid, sodium hydroxide, di-sodium hydrogen phosphate, sodium hydrogen phosphate, sodium chloride, ammonia hydroxyde, ammonium chloride, ammonium iodide, and 2-mercaptoethanol (all analytical grade) were purchased from Merck (Darmstadt, Germany). Ammonium formate (LC grade) was obtained from Sigma-Aldrich (Steinheim, Germany). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany). β -Glucuronidase from *Escherichia coli* K12 was obtained from Roche Diagnostics (Mannheim, Germany). Detectabuse™ XAD-2 extraction columns were purchased from Biochemical Diagnostics, Inc. (Edgewood, NY, USA). Milli-Q water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.2 Sample preparation

2.2.1. *Direct detection of sulfate conjugates by LC-MS/MS*

Extraction of sulfate conjugates was based on a previously described procedure.^[27] Briefly, 100 ng/mL of boldenone sulfate (internal standard, IS) were added to 5 mL aliquots of urine samples and the pH was made alkaline by adding 100 μ L of 5.3 M ammonium chloride solution (adjusted to pH 9.5 with ammonia). Then, sodium chloride (1 g) was added to promote salting-out effect and samples were extracted with 8 mL of ethyl acetate by shaking at 40 rpm for 20 min. After centrifugation (3000 g, 5 min), the organic layer was evaporated to dryness under a nitrogen stream in a water bath at 40 °C. The extracts were reconstituted

with 100 μ L of a mixture of Milli-Q water: ACN (9:1, v/v) and 10 μ L were analyzed by LC-MS/MS.

2. 2. 2. *Detection of metabolites excreted free and conjugated with glucuronic acid by GC-MS/MS*

Extraction of 4Cl-MTD metabolites for GC-MS/MS analysis was essentially performed as previously described.^[28] IS solution (25 μ L, methyltestosterone 10 μ g/mL) was added to urine samples (2.5 mL) together with 1 mL of sodium phosphate buffer (1 M, pH 7). Enzymatic hydrolysis was performed by adding 30 μ L of β -glucuronidase from *E. coli* and incubating the mixture at 55 °C for 1h. After the sample reached room temperature, 200 mg of NaHCO₃/Na₂CO₃ (1:2, w/w) buffer were added and the mixture was extracted with 6 mL of TBME by shaking at 40 mpm for 20 min. After centrifugation (3000 g, 5 min), the organic layer was separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C. The dry extracts were derivatized by adding 50 μ L of a mixture of a MSTFA/NH₄I/2-mercaptoethanol (1000:2:6, v|w|v) and incubating at 60 °C for 20 min. After incubation, the derivatized extracts were transferred to injection vials and 2 μ L were injected into the GC-MS/MS system.

2. 2. 3. *Detection of metabolites excreted free and conjugated with glucuronic acid by LC-MS/MS*

100 ng/mL of boldenone (IS) were added to urine samples (5 mL) which were passed through XAD-2 columns previously conditioned with 2 mL MeOH and 2 mL water. The column was washed with 2 mL water and the analytes were eluted with 2 mL MeOH. The organic extract was evaporated to dryness under a stream of nitrogen in a water bath at 50 °C and reconstituted with 1 mL of sodium phosphate buffer (0.2 M, pH 7). Enzymatic hydrolysis was

performed as described in the previous section. After the sample reached room temperature 250 μL of a 5% K_2CO_3 solution were added and the mixture was extracted with 6 mL of TBME by shaking at 40 rpm for 20 min. After centrifugation (3000 g, 5 min), the organic layer was separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 $^\circ\text{C}$. The dry extracts were reconstituted with 100 μL of a mixture of Milli-Q water:ACN (1:1, v/v) and 10 μL were analyzed by LC-MS/MS.

2.3 LC-MS/MS instrumental conditions

Chromatographic separations were carried out on a Waters Acquity UPLCTM system (Waters Corporation, Milford, MA) using an Acquity BEH C_{18} column (100 mm x 2.1 mm i.d., 1.7 μm particle size). The column temperature was set to 55 $^\circ\text{C}$, and the flow rate at 0.3 mL/min.

The study was conducted in a triple quadrupole (XEVOTM TQMS) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) interfaced to an Acquity UPLC system for the chromatographic separation (all from Waters Associates, Milford, Massachusetts, USA). Source conditions were fixed as follows: lens voltage, 0.2 V; source temperature, 120 $^\circ\text{C}$; cone gas flow rate, 50 L/h; desolvation gas flow rate, 1200 L/h. In positive mode the capillary voltage was set at 3 kV, whereas in negative mode the capillary voltage was set at 2.5 kV. Nitrogen was used as desolvation gas and argon was used as collision gas. The desolvation temperature was set at 550 $^\circ\text{C}$.

The mobile phase consisted of deionized water with 0.01% formic acid (solvent A) and ACN with 0.01% formic acid (solvent B). The following gradient elution was used for the separation of potential sulfate metabolites of 4Cl-MTD: from 0 to 0.5 min, 15% B; from 0.5 to 9 min, to 50% B; from 9 to 12 min, to 85% B; from 12 to 12.3 min, 85% B; from 12.3 to 12.5 min, to 15% B; from 12.5 to 15 min, 15% B.

For the detection of potential sulfate metabolites of 4Cl-MTD, data acquisition was performed using a selected reaction monitoring (SRM) method (Table 1, method 1).

2.4 GC-MS/MS instrumental conditions

The study was performed on an Agilent 7890A gas chromatograph equipped with a 7693 autosampler, a split/splitless capillary inlet and an Agilent 7000A Series Triple Quadrupole GC-MS/MS. The whole system was controlled by the MassHunter workstation software (revision B.04.00). The GC was equipped with a capillary column (HP-Ultra1 16.5 m × 0.2 mm id with a 0.11 µm film thickness) from J&W Scientific (Folsom, California, USA). The gas chromatograph temperature was ramped as follows: initial temperature 185 °C, increased at 25 °C/min to 230 °C, then at 10 °C/min to 290 °C, then at 70 °C/min to 310 °C, and maintained at the final temperature for 2 min. The injector and transfer lines were kept at 280 °C. A 2 µL aliquot of the final extract was injected in split mode (split ratio 1:10). Nitrogen was used as collision gas at a flow rate of 1.5 mL/min, and helium as a quenching gas at a flow rate of 2.25 mL/min. The electron ionization source was kept at 230 °C and the quadrupoles at 150 °C. Data acquisition was performed in SRM mode, including ion transitions of all 4Cl-MTD metabolites described up to now (Table 1, method 3).^[5]

2.5 Identification of the sulfate metabolites

For metabolite characterization, LC fractionation of urine samples was performed. Sample preparation described above for the extraction of sulfate conjugates (section 2.2.1) was applied to 3 aliquots of 5 mL of a post-administration urine sample. The organic phases of the three replicates were combined, evaporated to dryness, reconstituted (100 µL Milli-Q water:ACN (90:10, v/v)) and injected (10 µL) into the LC system. A T-device was connected after the chromatographic column to deviate 50% of the eluent to the fraction tube and 50% to

the MS. The same gradient elution as described in Section 2.3 was used. Data acquisition was performed in SRM mode by monitoring the most sensitive ion transition for each metabolite. Different fractions corresponding to the expected retention time ($RT \pm 0.2$ min) of the metabolites were manually collected and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C.

Afterwards, the fractions were subjected to hydrolysis of the sulfate metabolites using a solvolysis procedure.^[29] For that purpose, 500 ng of 17 β -boldenone sulfate were added to every fraction, fractions were reconstituted with 4 mL of ethyl acetate/methanol/sulfuric acid (80:20:0.06, v|v|v) and incubated at 55 °C for 2 h. After incubation, samples were neutralized with 60 μ L of a 1 M NaOH solution and evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 1 mL of sodium phosphate buffer (0.2 M, pH 7) and 250 μ L of 5 % K_2CO_3 solution were added. A liquid-liquid extraction was performed with 5 mL of TBME by vortex mixing for 1 min. After centrifugation (3000 g, 5 min), the organic layers were separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 °C.

Sample were analyzed by LC-MS/MS and/or GC-MS/MS. For LC-MS/MS analysis, the dry extracts were reconstituted with 100 μ L of a mixture of Milli-Q water:ACN (1:1, v|v) and 10 μ L were injected into the system. For characterization of the sulfate metabolite S8a, after fraction collection and solvolysis, a different gradient was used: from 0 to 0.5 min, 15% B; from 0.5 to 3 min, to 50% B; from 3 to 12 min, to 85%; from 12 to 12.3 min, to 85% B; from 12.3 to 12.5 min, 15% B; from 12.5 to 15 min, 15% B. Data acquisition was performed in SRM mode, using a method with characteristic transitions of the released phase I metabolite (Table 1, method 2).

For GC-MS/MS analyses, the dry extracts were derivatized as previously indicated (section 2.2.2) and same equipment and conditions as mentioned in Section 2.4 were used.

Deconjugation efficiency was checked by the occurrence of a peak corresponding to free 17 β -boldenone in the analysis.

2.6 Excretion study samples

Urine samples obtained from two excretion studies involving the administration of a single oral dose of 4CI-MTD to healthy male volunteers were analyzed. The clinical protocol was approved by the Local Ethical Committee (CEIC-IMAS, Institut Municipal d'Assistència Sanitària, Barcelona, Spain). Urine samples were collected before administration and after administration. Subject A (Caucasian, 49 years, 85 kg) was administered a single oral dose of 20 mg and urine fractions at 0-4 h, 4-8 h, 8-12 h, 12-24 h, 24-36 h, 48-56 h and 72-84 h were collected. Study B (Caucasian, 24 years, 78 kg) consisted of a single oral dose of 5 mg and collection of the urines was as follow: 0-12 h, 12-24 h, 24-48 h, 48-72 h, and daily spot morning urines from day 5 to day 8 after administration. Urine samples were stored at -20 °C until analysis.

3 Results and discussion

3.1 Method development

In the present study, the direct detection of sulfate metabolites of 4Cl-MTD was evaluated by LC-MS/MS. A targeted approach based on a SRM method which has been previously reported as a more sensitive strategy compared to untargeted methods (NL or precursor ion scan acquisition modes)^[13] was applied to pre- and post-administration samples. A theoretical SRM method was developed in negative mode because of the higher ionization efficiency of these metabolites when working under this mode (Table 1, method 1).^[30] The theoretical ion transitions were designed based on common ionization and fragmentation behavior of sulfate conjugates and on specific neutral losses (NL) of compounds with related chemical structure (e.g. boldenone or clostebol).^[9,19]

As precursor ions, the $[M-H]^-$ ions of potential sulfate metabolites were calculated considering all phase I metabolites described up to now.^[5,22-26] Metabolites **1**, **6** and **13** (Figure 1) were excluded from the method since they only contain a hydroxyl moiety in their structure which is a tertiary 17 β -hydroxy group. Sulfation at the 17 β -hydroxy group is sterically influenced and spontaneously decomposes in urine to yield several degradation products.^[3,31,32] Therefore, they are not expected to appear in urine.

For the rest of metabolites, several structural considerations were taken into account for the selection of the product ions. Firstly, the characteristic product ion of sulfate metabolites (m/z 97)^[30,33] was selected for all metabolites. Additionally, specific product ions based on the chemical structure of the A-ring of the metabolites were also selected. On the one hand, for metabolites with a 3-keto-4-Cl-1,4-diene structure the ion transitions resulting from the NL of a methyl group (15 Da) were incorporated.^[9,11] On the other hand, for those metabolites with a reduced double bond in C4-C5, ion transitions resulting from the NL of HCl were added.^[19]

Finally, the same ion transitions considering ^{37}Cl isotope were also added into the method (Table 1, method 1).

3.2 Detection of 4Cl-MTD metabolites excreted as sulfate conjugates

The developed SRM method was applied to pre and post-administration samples. Among the 12 potential sulfate metabolites included in the method, peaks at the theoretical ion transitions for metabolites **2**, **7**, **8**, **11** and **12** were detected in post-administration samples together with many other peaks of lower abundance (Table 2). Two peaks were detected at the ion transitions expected for metabolite **8**. No peaks at the same retention time (RT) were observed in pre-administration samples.

The molecular mass (MM), RTs and corresponding phase I metabolites are listed in Table 2. For all the metabolites, the ion transitions of specific losses (loss of the methyl group or loss of HCl) showed higher signal to noise ratios compared to the ion transitions to the common product ion at m/z 97. Only metabolite S7, although having better detectability with the ion transition of the NL of HCl, could also be detected using the ion transition to m/z 97. For the rest of metabolites this ion transition was highly interfered by matrix components, probably endogenous sulfates, preventing their detection. Thus, the use of specific losses improved the detection of these metabolites using SRM analysis as previously described.^[9,19] The presence of other sulfates (e.g. sulfate of metabolite **10**) without specific NLs cannot be discarded.

3.3 Characterization of sulfate metabolites

The characterization of the sulfate metabolites was performed taking into consideration the information obtained in the product ion mass spectra (Figure 2). Depending on their mass spectrometric behavior, metabolites could be divided into two groups. Metabolites S2, S8a and S8b (group 1) showed two main product ions corresponding to the NL of 15 Da

(indicating a conjugated A-ring) and the characteristic product ion at m/z 97 (Figure 2, group 1). Metabolites from group 2 (S7, S11 and S12) were also characterized by two different product ions corresponding to the NL of HCl (indicating a totally or partially reduced A-ring) and the product ion at m/z 97 (Figure 2, group 2). As same as for other sulfate steroids, the ion at m/z 80 was also observed for some of the metabolites.^[30]

Due to the limited structural information obtained by the product ion spectra of unaltered sulfates, an additional strategy was performed consisting of the collection of the LC fraction of the peak of the sulfate metabolite, subsequent solvolysis to release the phase I metabolite, and analysis by LC-MS/MS or GC-MS/MS. Some of the phase I metabolites released after solvolysis of the sulfates were not ionizable using electrospray ionization (those lacking a conjugated 3-keto function in the structure)^[30] and, for this reason, identification analysis had to be performed using GC-MS/MS.

3.3.1. *Metabolite S2*

The ion transitions selected to monitor metabolite S2 were predicted based on either metabolite **2** or metabolite **3** which only differ in the position of the hydroxyl group (Figure 1). The results obtained from the product ion mass spectra revealed the presence of only two product ions corresponding to the loss of 15 Da and the ion at m/z 97 (Figure 2). Additional product ions were not observed. Metabolite **2** is commercially available as standard and the results of the analysis by GC-MS/MS of the standard compound after derivatization were compared with the results of the analysis by GC-MS/MS of the LC fraction corresponding to the sulfate metabolite S2 after solvolysis, extraction and derivatization (Figure 3). Relative retention times (RRT) (1.367 vs. 1.363) and ion ratios were very similar indicating that sulfate S2 is the sulfate conjugate of metabolite **2** (6 β -hydroxy-4Cl-MTD). Metabolite **2** contains two hydroxyl groups in its structure. Since spontaneous degradation is expected for sulfates at 17-

position, it can be predicted that metabolite S2 has the sulfate in position 6. For the first time, metabolite **2** has been detected as a sulfate conjugate. Up to now, its excretion as a free metabolite had only been reported.^[24,34]

3.3.2. *Metabolite S7*

The ion transitions used to monitor metabolite S7 were selected taken into account the structure of metabolite **7**. The product ion scan results obtained after LC-MS/MS analysis revealed the expected behavior for metabolite **7**, two main product ions corresponding to the loss of HCl and the hydrogen sulfate moiety (m/z 97) (Figure 2). Regarding the GC-MS/MS results, no peak was detected in the whole chromatographic window of the characteristic ion transitions of metabolite **7** after GC-MS/MS analysis of the extract obtained after LC fractionation of S7 solvolysis and derivatization. The presence of the 17 β -boldenone peak demonstrated the deconjugation efficiency and so, the absence of the metabolite peak is probably due to its low concentration. Although LC-MS/MS data seems to be consistent with the assignment of S7 as sulfation of metabolite **7**, additional data is required to ultimately confirm the structure of this metabolite. Metabolite **7** contains three hydroxyl groups in its structure, being two of them (in position 3 and 6) possible sites of sulfation.

3.3.3. *Metabolites S8a and S8b*

Metabolites S8a and S8b had the same MM (412 Da), however the signal intensity was higher for S8a and, for this reason, the identification work for these metabolites focused on metabolite S8a.

The selected ion transitions to detect the sulfate metabolite S8a considered metabolite **8** as the most feasible structure for this sulfate conjugate. To confirm the proposed structure, product ion scan of S8a was acquired. As expected, the results only showed the product ion resulting from the loss of 15 Da and the product ion at m/z 97. No other product ions characteristic of this metabolite were observed (Figure 2). Additionally the LC fraction of the peak of S8a was

collected and, after applying a solvolysis procedure, samples were analyzed by LC-MS/MS in SRM mode. The theoretical ion transitions for the LC-MS/MS detection of metabolite **8** were based on the previously reported metandienone metabolite with related chemical structure.^[6] They corresponded to the NL of formaldehyde (30 Da), characteristic of hydroxymethyl groups, and to the ions m/z 155 and m/z 181 which indicate an intact 3-keto-4-Cl-1,4-diene structure (Table 1, method 2).^[6] Results are presented in Figure 4. The phase I metabolite released after solvolysis of S8a showed a peak at 8.24 min in all transitions (Figure 4, C). For comparison purposes, 4Cl-MTD pre- and post-administration samples were hydrolyzed with β -glucuronidase, extracted and analyzed by LC-MS/MS using the same method. A peak at the same RT (8.24 min) was obtained in the post-administration sample (Figure 4, B). No peaks were observed in the blank sample (Figure 4, A). Relative abundances obtained in both peaks were very similar (Figure 4) indicating that the peaks detected after solvolysis of S8a and after enzymatic hydrolysis of metabolite **8** glucuronide corresponded to the same compound.

Therefore, metabolite S8a was identified as 4-chloro-18-nor-17 β -hydroxymethyl-17 α -methylandrost-1,4,13-triene-3-one conjugated with sulfate.

Given the MM and the product ions, the most feasible structure for metabolite S8b is the second isomer of the same metabolite. Nevertheless, the ultimate confirmation of S8a and S8b structure would require synthesis of the compounds and comparison of analytical data with that obtained from the compound detected in urine. Metabolite S8a has analogue structure of the long-term sulfate metabolite identified for metandienone, another 17 β -hydroxy-17 α -methyl steroid.^[11]

3.3.4. *Metabolite S11*

Ion transitions 415→97 and 417→97 were selected as common for metabolites **10**, **epi10** and **11**. Metabolites S11 also gave signal to the ion transitions 415→379 and 417→379 which were selected as specific of metabolite **11**. Therefore, sulfation of metabolite **11** was found to be the most feasible structure of S11. The LC-MS/MS product ion scan was in agreement with the proposed structure since only ions at m/z 97 and m/z 379 were observed. This last ion is indicative of a reduction in C4-C5. Despite obtaining a proper solvolysis yield (demonstrated by the presence of the released 17 β -boldenone), no peaks were observed in the characteristic ion transitions of metabolites **11** after GC-MS/MS analysis of the phase I metabolite released after solvolysis of S11. Thus, the structure of the metabolite could not be confirmed.

3.3.5. *Metabolite S12*

Theoretical transitions for 4-chloro-18-nor-17 ζ -hydroxymethyl-17 ζ -methyl-5 ζ -androst-13-en-3 ζ -ol were selected for the detection of S12. The LC-MS/MS product ion mass spectra results were in agreement with this assignment since only two main product ions corresponding to the NL of HCl and the product ion at m/z 97 were observed (Figure 2). Similar to S11, GC-MS/MS analysis, revealed no peaks in the whole chromatographic window of the characteristic ion transitions of metabolites **12** and **epi12** after analysis of the phase I metabolite released after solvolysis of S12, probably due to the low concentration of the metabolite.

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

An SRM method for the qualitative analysis of 4Cl-MTD sulfate metabolites was optimized. Cone voltages 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 method, cone voltages for metabolites **8**, **2/3** and **4/5** were set at 60 V whereas the rest of

parameters stayed the same as per Method 1 (table 1). Afterward, the method was applied to excretion study samples with the aim to evaluate the detection times of the different metabolites. The absence of any interfering substance at the RT of the analytes was verified by analysis of ten blank urines.

Detection times of the different sulfate metabolites are detailed in Figure 5. Metabolite S2 had the lowest retrospectivity in both studies, being 12 h in study A and 5 days in study B. The other five metabolites (S7, S8a, S8b, S11 and S12) were detected up to the last collected sample in both excretion studies (84 h and 8 days, respectively). Figure 6 shows the SRM chromatograms for these five metabolites for a pre-administration and a post-administration sample (day 8) of study B. Remarkably, metabolite S7 was only detected after the first 4h in study A and metabolite S12 was not detected during the first 48h in both excretion studies (Figure 5). Based on the abundances of the different metabolites obtained for volunteer B, the sulfate metabolite S12 would probably have the longest detection times after 4Cl-MTD administration (Figure 6).

3.5 Evaluation of excretion profiles of free and glucuronide metabolites by GC-MS/MS

For comparison purposes, the samples from both excretion studies were analyzed by using the commonly employed GC-MS/MS screening method (Table 1, method 3), after enzymatic hydrolysis with β -glucuronidase and derivatization. This method includes the previously described metabolites of 4Cl-MTD excreted free and conjugated with glucuronic acid (Figure 1) ^[5]. The detection times of these metabolites are detailed in Figure 5. Most of the metabolites detected in the glucuronide fraction were detected up to the last collected samples, 84 h and 8 days for studies A and B, respectively. Metabolite **8** has been detected for longer excretion times in previous studies;^[3,26] however 40 mg and 15 mg were administered

instead of the 5 mg administered in our study B. As observed for the sulfate metabolites, some of the metabolites were not detected during the first hours after administration (metabolites **7**, **12** and **epi12** in both studies; metabolite **9** in study A; metabolite **11** in study B). Further, metabolite **9** was not detected in any of the samples of study B. As previously reported, these results show a considerable inter-individual variability in the metabolism of 4Cl-MTD.^[5]

Most of the metabolites could be detected up to the last collected samples in both excretion studies. These results do not allow us to predict which would be the metabolites detected for the longest period of time. Figure 5 summarizes the results of each of the phase I metabolites (excreted free or coming from a glucuronide) and the sulfate conjugates. Metabolite **2** had identical detection times as its respective sulfate metabolite (S2) for volunteer B. For metabolites **7**, **10**, **epi10**, **11**, **12** and **epi12**, detectability was up to the last collected samples for both, the hydrolyzed or free metabolites and their respective sulfate metabolites (S7, S11 and S12). However, based on our previous experience and the results obtained for sulfate metabolite S8a in comparison with its corresponding glucuronide (metabolite **8**), which show a better detectability of the sulfate, we strongly believe sulfates will have longer detection times with respect to their corresponding glucuronides.^[9-11] Additional excretion studies with longer collection times will be necessary to further investigate the excretion profiles of the metabolites in order to define the best target metabolites during the whole excretion period.

4 Conclusions

The sulfate fraction of 4Cl-MTD has been studied for the first time. Six 4Cl-MTD sulfate metabolites have been described in human urine. Five of the sulfate metabolites are detected in urine up to the last collected samples demonstrating the high potential of these metabolites to be 4Cl-MTD long-term metabolites. Excretion studies with longer collection periods have

to be performed to determine which of the metabolites provides better detectability after 4Cl-MTD misuse.

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References

- [1] World Anti-Doping Agency (WADA). The 2016 Prohibited List. International Standard. Available from: <https://wada-main-prod.s3.amazonaws.com/resources/files/wada-2016-prohibited-list-en.pdf>. (accessed June, 2016).
- [2] World Antidoping Agency (WADA). 2014 Adverse Analytical Findings and Atypical Findings Reported by Accredited Laboratories. Available from: <https://www.wada-ama.org/en/resources/laboratories/anti-doping-testing-figures#.VBmQFhZNrzB>. (accessed January, 2016).
- [3] W. Schanzer. Metabolism of anabolic androgenic steroids. *Clin. Chem.* **1996**, 42, 1001.
- [4] P. Van Eenoo, F.T. Delbeke. Metabolism and excretion of anabolic steroids in doping control--new steroids and new insights. *J. Steroid Biochem. Mol. Biol.* **2006**, 101, 161.
- [5] T. Sobolevsky, G. Rodchenkov. Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine. *J. Steroid Biochem. Mol. Biol.* **2012**, 128, 121.
- [6] W. Schanzer, H. Geyer, G. Fussholter, N. Halatcheva, M. Kohler, M.K. Parr, S. Guddat, A. Thomas, M. Thevis. Mass spectrometric identification and characterization of a new long-term metabolite of metandienone in human urine. *Rapid Commun Mass Spectrom.* **2006**, 20, 2252.
- [7] O.J. Pozo, W. Van Thuyne, K. Deventer, P. Van Eenoo, F.T. Delbeke. Elucidation of urinary metabolites of fluoxymesterone by liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry. *J. Mass Spectrom.* **2008**, 43, 394.
- [8] C. Gomez, O.J. Pozo, A. Fabregat, J. Marcos, K. Deventer, P. Van Eenoo, J. Segura, R. Ventura. Detection and characterization of urinary metabolites of boldione by LC-MS/MS. Part I: Phase I metabolites excreted free, as glucuronide and sulfate conjugates, and released after alkaline treatment of the urine. *Drug Test. Anal.* **2012**, 4, 775.
- [9] C. Gomez, O.J. Pozo, H. Geyer, J. Marcos, M. Thevis, W. Schanzer, J. Segura, R. Ventura. New potential markers for the detection of boldenone misuse. *J. Steroid Biochem. Mol. Biol.* **2012**, 132, 239.

- [10] C. Gomez, O.J. Pozo, J. Marcos, J. Segura, R. Ventura. Alternative long-term markers for the detection of methyltestosterone misuse. *Steroids*. **2013**, 78, 44.
- [11] C. Gomez, O.J. Pozo, L. Garrostras, J. Segura, R. Ventura. A new sulphate metabolite as a long-term marker of metandienone misuse. *Steroids*. **2013**, 78, 1245.
- [12] M. Thevis, T. Piper, S. Horning, D. Juchelka, W. Schanzer. Hydrogen isotope ratio mass spectrometry and high-resolution/high-accuracy mass spectrometry in metabolite identification studies: detecting target compounds for sports drug testing. *Rapid Commun Mass Spectrom*. **2013**, 27, 1904.
- [13] A. Fabregat, O.J. Pozo, J. Marcos, J. Segura, R. Ventura. Use of LC-MS/MS for the open detection of steroid metabolites conjugated with glucuronic acid. *Anal. Chem*. **2013**, 85, 5005.
- [14] W. Schanzer, S. Guddat, A. Thomas, G. Opfermann, H. Geyer, M. Thevis. Expanding analytical possibilities concerning the detection of stanozolol misuse by means of high resolution/high accuracy mass spectrometric detection of stanozolol glucuronides in human sports drug testing. *Drug Test Anal*. **2013**, 5, 810.
- [15] J. Lu, M. Fernandez-Alvarez, S. Yang, G. He, Y. Xu, R. Aguilera. New clostebol metabolites in human urine by liquid chromatography time-of-flight tandem mass spectrometry and their application for doping control. *J Mass Spectrom*. **2015**, 50, 191.
- [16] J. Lu, M. Fernandez-Alvarez, S. Yang, G. He, Y. Xu, R. Aguilera. New potential biomarkers for mesterolone misuse in human urine by liquid chromatography quadrupole time-of-flight mass spectrometry. *J Mass Spectrom*. **2015**, 50, 153.
- [17] P. Kiouisi, Y.S. Angelis, A.G. Fragkaki, W. Abushareeda, M. Alsayrafi, C. Georgakopoulos, E. Lyris. Markers of mesterolone abuse in sulfate fraction for doping control in human urine. *J Mass Spectrom*. **2015**, 50, 1409.
- [18] A.G. Fragkaki, Y.S. Angelis, P. Kiouisi, C.G. Georgakopoulos, E. Lyris. Comparison of sulfo-conjugated and gluco-conjugated urinary metabolites for detection of methenolone misuse in doping control by LC-HRMS, GC-MS and GC-HRMS. *J Mass Spectrom*. **2015**, 50, 740.
- [19] G. Balcells, O.J. Pozo, L. Garrostras, A. Esquivel, X. Matabosch, A. Kotronoulas, J. Joglar, R. Ventura. Detection and characterization of clostebol sulfate metabolites in Caucasian population. *J Chromatogr B*. **2016**, 1022, 54.
- [20] E. Tudela, K. Deventer, L. Geldof, P. Van Eenoo. Urinary detection of conjugated and unconjugated anabolic steroids by dilute-and-shoot liquid chromatography-high resolution mass spectrometry. *Drug Test Anal*. **2014**, 7, 95.
- [21] G. Balcells, O.J. Pozo, A. Esquivel, A. Kotronoulas, J. Joglar, J. Segura, R. Ventura. 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. *J Chromatogr A*. **2015**, 1389, 65.
- [22] K. Schubert, K. Wehrberger. [Metabolism of steroid drugs. II. Isolation and identification of metabolites of 4-chlor-17 alpha-methyl-17 beta-hydroxy-1,4-androstadien-3-one]. *Endokrinologie*. **1970**, 55, 257.
- [23] K. Schubert, G. Schumann. [Metabolism of steroid drugs. IV. Isolation and identification of a dihydroxylated metabolite of 4-chlorine-17 alpha-methyl-17 beta-hydroxy-1,4-androstadien-3-on]. *Endokrinologie*. **1970**, 56, 172.
- [24] H.W. Durbeck, I. Buker, B. Scheulen, B. Telin. GC and capillary column GC/MS determination of synthetic anabolic steroids. II. 4-chloro-methandienone (oral turinabol) and its metabolites. *J Chromatogr Sci*. **1983**, 21, 405.
- [25] W. Schanzer, S. Horning, G. Opfermann, M. Donike. Gas chromatography/mass spectrometry identification of long-term excreted metabolites of the anabolic steroid 4-chloro-1,2-dehydro-17alpha-methyltestosterone in humans. *J Steroid Biochem Mol Biol*. **1996**, 57, 363.

- [26] M.K. Parr, G. Fußhöller, M. Gütschow, C. Hess, W. Schanzer, GC-MS(/MS) investigations on long-term metabolites of 17-methyl steroids, Recent Advances in Doping Analysis: Proceedings of Manfred Donike Workshop; Schänzer W, Geyer H, Gotzmann A, Mareck U, Köln, **2010**.
- [27] R. Ventura, M. Roig, N. Montfort, P. Saez, R. Berges, J. Segura. High-throughput and sensitive screening by ultra-performance liquid chromatography tandem mass spectrometry of diuretics and other doping agents. *Eur J Mass Spectrom (Chichester, Eng)*. **2008**, 14, 191.
- [28] M.A. Delgadillo, L. Garrosta, O.J. Pozo, R. Ventura, B. Velasco, J. Segura, J. Marcos. Sensitive and robust method for anabolic agents in human urine by gas chromatography-triple quadrupole mass spectrometry. *J. Chromatogr. B*. **2012**, 897, 85.
- [29] S. Torrado, M. Roig, M. Farre, J. Segura, R. Ventura. Urinary metabolic profile of 19-norsteroids in humans: glucuronide and sulphate conjugates after oral administration of 19-nor-4-androstenediol. *Rapid Commun Mass Spectrom*. **2008**, 22, 3035.
- [30] C. Gomez, A. Fabregat, O.J. Pozo, J. Marcos, J. Segura, R. Ventura. Analytical strategies based on mass spectrometric techniques for the study of steroid metabolism. *Trends Anal. Chem*. **2013**, 53, 106.
- [31] H. Bi, R. Masse. Studies on anabolic steroids--12. Epimerization and degradation of anabolic 17 beta-sulfate-17 alpha-methyl steroids in human: qualitative and quantitative GC/MS analysis. *J Steroid Biochem Mol Biol*. **1992**, 42, 533.
- [32] W. Schanzer, G. Opfermann, M. Donike. 17-Epimerization of 17 alpha-methyl anabolic steroids in humans: metabolism and synthesis of 17 alpha-hydroxy-17 beta-methyl steroids. *Steroids*. **1992**, 57, 537.
- [33] L. Yi, J. Dratter, C. Wang, J.A. Tunge, H. Desaire. Identification of sulfation sites of metabolites and prediction of the compounds' biological effects. *Anal. Bioanal. Chem*. **2006**, 386, 666.
- [34] W. Schanzer, S. Horning, M. Donike. Metabolism of anabolic steroids in humans: synthesis of 6 beta-hydroxy metabolites of 4-chloro-1,2-dehydro-17 alpha-methyltestosterone, fluoxymesterone, and metandienone. *Steroids*. **1995**, 60, 353.

Table 1. Mass spectrometric methods used during the study. Parameters: precursor ion (PI), product ion (DI), cone voltage (CV), collision energy (CE). MM, molecular mass. For the structure of the metabolites see Figure 1.

Method 1 LC-MS/MS	Metabolite	MM (Da)	Ionization mode	PI (m/z)	DI (m/z)	CV (V)	CE (eV)
SRM	8 + sulfate	412	ESI -	411	97	40	40
				411	396	40	30
				413	97	40	40
				413	398	40	30
	10/epi10 + sulfate	416	ESI -	415	97	40	40
				417	97	40	40
	11 + sulfate	416	ESI -	415	97	40	40
				415	379	40	30
				417	97	40	40
				417	379	40	30
	12/epi12 + sulfate	418	ESI -	417	97	40	40
				417	381	40	30
				419	97	40	40
				419	381	40	30
	2/3 + sulfate	430	ESI -	429	97	40	40
				429	414	40	30
				431	97	40	40
				431	416	40	30
	9 + sulfate	436	ESI -	435	97	40	40
				435	399	40	30
				437	97	40	40
				437	399	40	30
	4/5 + sulfate	446	ESI -	445	97	40	40
				445	430	40	30
				447	97	40	40
				447	432	40	30
	7 + sulfate	448	ESI -	447	97	40	40
				447	411	40	30
				449	97	40	40
				449	411	40	30
	Boldenone sulfate (IS)	366	ESI -	365	350	60	30
Method 2 LC-MS/MS	Metabolite	MM (Da)	Ionization mode	PI (m/z)	DI (m/z)	CV (V)	CE (eV)
SRM	8	332	ESI+	333	155	20	20
				333	181	20	15
				333	303	20	15
				335	157	20	20
				335	183	20	15
				335	305	20	15
	Boldenone (IS)	286	ESI+	287	121	25	25
Method 3 GC-MS/MS	Metabolite	MM (Da)		PI (m/z)	DI (m/z)		CE (eV)
SRM	2	350		315	241		15
				315	227		20
				315	163		20
				243	164		25
	8	332		476	373		14
				476	133		16
	7	368		658	244		15
				656	244		17
	9	356		572	231		16
				572	143		22
	11	336		287	185		12
				289	185		20
				377	185		22
				379	185		15

12/epi12	338	379	343	7
		379	253	10
		381	343	10
		381	253	10
10/epi10	336	377	149	10
		379	149	13
		287	125	22
		289	125	10
Methyltestosterone (IS)	302	446	301	20

Table 2. Sulfate metabolites of 4Cl-MTD detected in post-administration samples. MM; molecular mass, RT; retention time.

Code	Corresponding phase I metabolite ^a	MM (Da)	RT (min)
S2	metabolite 2	430	4.80
S7	metabolite 7	448	4.57
S8a	metabolite 8	412	6.95
S8b		412	6.22
S11	metabolite 11	416	6.31
S12	metabolite 12	418	7.35

^a The sulfate metabolite could have the exact same chemical structure of the corresponding phase I metabolite or could be an isomer of it.

FIGURE LEGENDS

Figure 1. Scheme of metabolism of 4Cl-MTD based on literature; (1): 4Cl-MTD, (2): 6 β -hydroxy-4Cl-MTD, (3): 16 β -hydroxy-4Cl-MTD, (4): 6 β ,16-dihydroxy-4Cl-MTD, (5): 6 β ,12-dihydroxy-4Cl-MTD, (6): epi-4Cl-MTD, (7): 4-chloro-3 α ,6 β ,17 β -trihydroxy-17 α -methyl-androst-1-en-16-one, (8): 4-chloro-18-nor-17 β -hydroxymethyl,17 α -methylandrosta-1,4,13-trien-3-one, (9): 4-chloro-17 α -methyl-5 β -androstan-3 α ,16,17 β -triol, (10): 4-chloro-18-nor-17 β -hydroxymethyl,17 α -methylandrosta-4,13-dien-3 α -ol, (**epi10**): 4-chloro-18-nor-17 α -hydroxymethyl,17 β -methylandrosta-4,13-dien-3 α -ol, (**11**): 4-chloro-18-nor-17 β -hydroxymethyl,17 α -methyl-5 β -androsta-1,13-dien-3 α -ol, (**12**): 4-chloro-18-nor-17 β -hydroxymethyl,17 α -methyl-5 β -androst-13-en-3 α -ol, (**epi12**): 4-chloro-18-nor-17 α -hydroxymethyl,17 β -methyl-5 β -androst-13-en-3 α -ol, (**13**): Δ^6 -4-chloro-metandienone.

Figure 2. Product ion mass spectra of $[M-H]^-$ of the sulfate metabolites in ESI negative mode grouped by their structure; group 1, conjugated A-ring (CV= 60V; CE= 30eV); and group 2, partially/totally reduced A-ring (CV= 40V; CE= 30eV).

Figure 3. Identification by GC-MS/MS of the phase I metabolites released after solvolysis of metabolite S2. SRM chromatograms of the bis-TMS derivative of metabolite **2** of: A, blank urine after β -glucuronidase hydrolysis, liquid-liquid extraction and derivatization; B, reference material of metabolite **2**; and C, sample collected after 4CI-MTD administration after direct extraction of sulfate metabolites, LC fraction collection, solvolysis and derivatization.

Figure 4. Identification by LC-MS/MS of metabolite **8** released after solvolysis of metabolite S8a. SRM chromatograms and relative abundances (RA) of characteristic ion transitions of metabolite **8**: pre-administration (A) and post-administration (B) sample after hydrolysis with β -glucuronidase and liquid-liquid extraction; and C, post-administration sample after direct extraction of the sulfate metabolite, fraction collection and solvolysis to release the phase I metabolite from the sulfate S8a.

Figure 5. Detection times of the different metabolites of 4Cl-MTD obtained for the two excretion studies using GC-MS/MS analysis after enzymatic hydrolysis and derivatization (metabolites 2, 7, 9, 8, 10, epi10, 11, 12 and epi12), or using direct LC-MS/MS analysis of sulfate metabolites (metabolites S2, S7, S8a, S8b, S11 and S12).

Figure 6. LC-MS/MS results: SRM chromatograms (transitions corresponding to S7, S8a/S8b, S11 and S12) of a pre-administration sample and a post-administration sample (day 8) corresponding to excretion study B.