Detection and characterization of clostebol sulfate metabolites in Caucasian population

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Abstract

Anabolic androgenic steroids (AAS) are synthetic testosterone derivatives which undergo extensive metabolism in man. Differences in the excretion of phase II metabolites are strongly associated with inter-individual and inter-ethnic variations. Sulfate metabolites have been described as long-term metabolites for some AAS. Clostebol is the 4-chloro derivative of testosterone and the aim of the present study was the evaluation of clostebol sulfate metabolites in Caucasian population by LC-MS/MS technology. Clostebol was orally administered to four healthy Caucasian male volunteers, and excretion study urines were collected up to 31 days. Several analytical strategies (neutral loss scan, precursor ion scan and selected reaction monitoring acquisitions modes) were applied to detect sulfate metabolites in post-administration samples. Sixteen sulfate metabolites were detected, five of them having detectability times above 10 days (S1a, S2a, S3b, S3g and S4b). Interestingly, metabolite S1a could be detected up to the last collected sample of all excretion studies and it was characterized by LC-MS/MS and GC-MS as 4ξ-chloro-5α-androst-3β-ol-17-one 3β-sulfate. Thus, monitoring of S1a improves the detection time of clostebol misuse with respect to the commonly monitored metabolites, excreted in the glucuronide fraction. Importantly, this new metabolite can be incorporated into recently developed LC-MS/MS screening methods base on the direct detection of phase II metabolites.

Keywords: Clostebol, sulfate metabolites, LC-MS/MS, doping analysis, anabolic steroid

1. Introduction

Anabolic androgenic steroids (AAS) are performance enhancing drugs prohibited in sports by the World Anti-Doping Agency (WADA) [1]. For many years, AAS have represented the most detected family of substances in doping control analyses [2]. For this reason, there is a continuous effort to identify metabolites detectable for the longest period of time in urine (the so-called long-term metabolites) to improve the detection capabilities of the misuse of AAS.

The main phase II metabolic reactions described for AAS are glucuronidation and sulfonation. Glucuronidation is the most predominant pathway and glucuronide metabolites have been traditionally studied after hydrolysis with β-glucuronidase enzymes and detection of the released phase I metabolites by gas chromatography-mass spectrometry (GC-MS) and, later, by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [3-7].. Recent studies have shown the potential of LC-MS/MS for the direct detection of sulfate metabolites of AAS [8-12]. Some of these sulfate conjugates, despite being minor metabolites and undetectable when using conventional methods, provide longer retrospectivity compared to the major metabolites excreted as glucuronoconjugates [8,9]. For this reason, it seems interesting to study sulfate metabolites of other AAS. In addition, there is a growing tendency to develop LC-MS/MS screening methods based on the direct detection of phase II metabolites that can easily incorporate new sulfate metabolites [13,14].

In this work we focused on the study of sulfate metabolites of clostebol (CLO) (Figure 1). CLO, the 4-chloro derivative of testosterone, is a synthetic AAS which is used by athletes to increase muscular mass and to improve performance. In 1996, Schanzer *et al.* reported the detection of a sulfate metabolite after CLO oral administration using indirect methods [3]. Recently, eight CLO sulfate metabolites were detected in samples from a Chinese male volunteer using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF). One of these metabolites was detectable in urine for up to 25 days [15]. It is well known that the urinary excretion of some steroid metabolites

depends on inter-ethnic variations. Genetic polymorphisms may alter the expression or the activity of specific metabolic enzymes [16,17], and it has been reported for sulfotransferases, being some of them ethnically distributed [18]. Differences in plasma concentrations of endogenous steroid sulfates have been described between ethnic groups and, therefore, the existence of differential metabolism of AAS among populations may not be excluded.

The aim of the present work was to study metabolites of CLO conjugated with sulfate in Caucasian population using LC-MS/MS. The long-term metabolite isolated from urine was characterised by LC-MS/MS and GC-MS.

2. Experimental

2.1. Chemicals and reagents

4-Androsten-4-chloro-17β-ol-3-one (CLO) was obtained from Steraloids (Newport, United States). 4-chloro-androst-4-en-3α-ol-17-one and internal standards (IS, 17β-boldenone sulfate, androsterone-d4 3-glucuronide (d4-And-G), etiocholanolone-d5 (d5-Et) and nandrolone-d3 17-sulfate (d3-NAN-S)) were supplied by NMI Australian Government (Pymble, Australia). Boldenone was obtained from Sigma (Steinheim, Germany) and 17α-methyltestosterone (MET) from Toronto Research Chemicals (Toronto, Canada).

Tert-butylmethyl ether (TBME, for analysis), ethyl acetate (LC grade), acetonitrile (ACN) (LC grade), methanol (MeOH) (LC grade), formic acid (LC-MS grade), ammonium formate, sodium hydrogen carbonate (NaHCO$_3$), sodium carbonate (Na$_2$CO$_3$), 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). *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). Milli Q water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.2. Sample preparation procedures

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

Extraction of sulfate conjugates was based on a previously described procedure [19]. Briefly, 20 µL of the IS solution (MET and d3-NAN-S at 1 µg/mL) were added to 5 mL aliquots of urine samples

and the pH was made alkaline by adding 100 µL of 5.3 M ammonia/ammonium chloride buffer pH 9.5. Then, sodium chloride (1 g) was added to promote salting-out effect and the samples were extracted with 8 mL of ethyl acetate by shaking at 40 movements per minute (mpm) for 20 min. After centrifugation (4000 rpm, 5 min), the organic layer was evaporated to dryness under a nitrogen stream in a water bath at 40 °C. The extract was re-dissolved into 200 µL of a solution of ACN:water (10:90, v/v). A volume of 10 µL was injected into the LC-MS/MS.

2.2.2. GC-MS/MS analysis of CLO metabolites

Extraction of CLO metabolites for GC-MS/MS analysis was essentially performed as previously described [20]. After the addition of 25 µL of the IS solution (MET at 10 µg/mL, d4-And-G at 19,2 µg/mL and d5-Et at 12 µg/mL), urine samples (2.5 mL) were vortex-mixed and 1 mL of sodium phosphate buffer (1 M, pH 7) was added. Enzymatic hydrolysis was performed by adding 30 µL of β-glucuronidase from *E. coli* and incubating the mixture at 55 ºC for 1h. After the sample reached room temperature, 200 mg of $NaHCO_3/Na_2CO_3$ solid buffer were added and the mixture was extracted with 6 mL of TBME by shaking at 40 mpm for 20 min. After centrifugation (4000 rpm, 5 min), the organic layer was separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 ºC. The dry extract was derivatized by adding 50 µL of a mixture of $MSTFA/NH_4I/2$-mercaptoethanol (1000:2:6, v|w|v) and incubating at 60 ºC for 20 min. After incubation, the derivatized extracts were transferred to injection vials and 2 µL were analysed by GC-MS/MS.

2.3. LC-MS/MS instrumental conditions

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

was 450 ºC, and the source temperature was 120 ºC. In positive ionization mode, the capillary voltage was set at 3.5 kV, whereas in negative mode the capillary voltage was set at 2.5 kV.

Chromatographic separations were carried out on a Waters Acquity UPLC™ system (Waters Corporation, Milford, MA, USA) using an Acquity BEH C18 column (100 mm x 2.1 mm i.d., 1.7 µm particle size). The column temperature was set to 45 °C, and the flow rate at 0.3 mL/min. The mobile phase consisted of 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 pattern (solvent B) was used for the separation of sulfate metabolites of CLO: 0 min, 20%; 4 min, 20%; 30 min, 40%; 32 min, 70%; 34 min, 95%; 36 min, 95%; 37 min, 20%; 40 min, 20%.

For the untargeted detection of sulfate metabolites of CLO, data acquisition was performed using five different strategies (Table 1): precursor ion scan (PI) methods of m/z 143 and m/z 131 in positive mode and PI method of m/z 97 in negative mode (Table 1, methods 1 and 2); neutral loss scan (NL) method of 80 Da in positive mode and, NL methods of 36 and 38 Da in negative mode (Table 1, methods 3 and 4); and theoretical selected reaction monitoring (SRM) method (Table 1, method 5). For the target detection of sulfate metabolites of CLO (S1, S2, S3, S4 and S5) an SRM method was optimized (Table 1, Method 6).

For fraction collection of metabolite S1a, a T-device was connected after the column deviating 50 % of the eluent to the fraction tube and 50 % to the MS/MS instrument. The previously described gradient pattern was used. Data acquisition was performed in SRM mode by monitoring the specific ion transition at m/z 403→367.

2.4. GC-MS instrumental conditions

GC-MS/MS analyses of conventional CLO metabolites were 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 (Agilent Technologies, Santa Clara, CA, USA). The GC was equipped with a capillary column HP-Ultra1 (16.5 m × 0.2 mm inner diameter with a 0.11 µm film thickness) from J&W Scientific (Folsom, CA, USA). The gas chromatograph temperature was ramped as follows: initial temperature 180 ℃, increased at 3 ℃/min to 230 ℃, then at 40 ℃/min to 310 ℃ and maintained at the final temperature for 3 min. The injector and transfer lines were kept at 280 ℃. 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 ℃ and the quadrupoles at 150 ℃. The target detection of CLO metabolites was achieved by acquisition in SRM mode (Table 1, method 7).

For metabolite identification an Agilent 7890A gas chromatograph equipped with a 7693 autosampler and an Agilent 5975C Series GC-MS (Agilent Technologies, Palo Alto, CA, USA) was used. Helium was used as the carrier gas at a constant pressure. A 3 µL aliquot of the final derivatized extract was injected into the system. Same capillary column and temperature programme to the ones mentioned above were used. Data acquisition was performed in scan mode (m/z 50-700).

2.5. Identification of sulfate metabolites

For metabolite characterization, LC fractionation of urine samples was performed. Sample preparation described above (Section 2.2.1) for the extraction of sulfate conjugates was applied to 2 aliquots of 5 mL of a post-administration urine sample. The organic phases obtained were combined, evaporated to dryness, reconstituted (200 µL) and injected in a single injection (10 µL). Fractions

corresponding to the collection time window (expected retention time (RT) ± 0.2 min) of the peaks of each metabolite were manually collected. LC fractions were evaporated to dryness under a stream of nitrogen.

Fractions were subjected to hydrolysis of the sulfate metabolites. For that purpose, 500 ng of 17β-boldenone sulfate were added to every fraction as IS, then, fractions were reconstituted with 4 mL of ethyl acetate/methanol/sulfuric acid (80:20:0.06, v|v|v) and incubated at 55 ℃ for 2 h [21]. After incubation, samples were neutralized with 60 µL 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 µL of 5 % K_2CO_3 solution were added. The extraction was performed with 5 mL of TBME by shacking at 40 mpm for 20 min. After centrifugation (4000 rpm, 5 min), the organic layers were separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 ℃.

Before GC-MS analysis, the dry extracts were derivatized as previously indicated (section 2.2.2). The derivatized extracts were transferred to injection vials and injected into the system.

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

2.6. Excretion studies

Urine samples obtained from four excretion studies (A, B, C and D) involving the oral administration of CLO to healthy volunteers (male, Caucasian) were analysed. Studies were performed according to a clinical protocol approved by the Local Ethical Committee (CEIC-IMAS, Institut Municipal d'Assistència Sanitària, Barcelona, Spain). The urine samples were collected before administration and after administration at different time periods. Study A consisted of 3 repeated doses of 15 mg

separated 12 hours, urine samples were collected as follows: 0-12h, 12-24h, 24-48h, 48-72h, and daily spot morning urines on day 5, 7 and 10 after the first dose. Subject B was administered a single oral dose of 20 mg and urine fractions at 0-12h, 12-24h, 24-36h and 36-48h were collected. Studies C and D consisted of a single oral dose of 10 mg and collection of urines was as follows: 0-12h, 12-24h, 24-36h, 36-48h, 48-60h, 60-72h, 72-84h, 84-96h and 96-108h and daily spot morning urines from day 6 to day 31 after administration. Urine samples were stored at -20 ℃ until analysis.

3. Results

3.1. LC-MS/MS strategies for the detection of CLO metabolites excreted as sulfate conjugates

Several analytical strategies were applied in order to directly detect CLO sulfate metabolites. Only peaks detected in post-administration samples and not present in pre-administration samples were considered as potential sulfate metabolites of CLO. First of all, two PI methods and one NL method were designed based on the common fragmentation behaviour of both, CLO metabolites and sulfate conjugates (Table 1, method 1-3). CLO and metabolites with unmodified A-ring exhibit an abundant $[M+H]^+$ in positive mode. Their CID spectra yield the ions m/z 131 and m/z 143. For this reason, PI methods of these typical product ions (Table 1, method 1) were applied to pre- and post-administration samples. On the other hand, all sulfate metabolites show the product ion m/z 97 (corresponding to HSO_4^-) when working in negative mode [9,22], and a PI method of m/z 97 in negative mode was also used (Table 1, method 2). Considering positive ionization mode, one of the most common fragments of steroid sulfates is the NL of 80 Da (loss of a sulfur trioxide group) [8,22]. Hence, a NL method of 80 Da was employed. After applying these three approaches, only the PI of m/z 97 showed one metabolite (S1a, Table 2) in the total ion ion chromatogram (TIC) of post-administration samples. When data of the PI of m/z 97 was evaluated using extracted ion chromatograms (XIC) of the expected metabolites, sixteen sulfate metabolites were detected (Table 2). On the contrary, PI methods of m/z 131 and m/z 143 and NL method of 80 Da in positive mode did not allow for the detection of any metabolites.

In a second step, product ion scans of $[M-H]^-$ were performed with the aim to find out additional ions or losses common to the detected metabolites. The results revealed an abundant product ion corresponding to a loss of 36 Da which matched with either two molecules of H_2O or a NL of HCl. Product ion scans of $[M+2-H]^-$ showed a NL of 38 Da which confirmed that the observed NL corresponded to HCl. For the first time, a characteristic product ion corresponding to the NL of HCl

in negative mode has been described for sulfate metabolites containing Cl. This loss yields a more specific ion compared to the common ion m/z 97. Figure 2 shows the product ion mass spectra of [M-H]$^-$ and [M+2-H]$^-$ of one of the metabolites.

Based on these results, two additional methods (NL and SRM) were developed (Table 1, method 4 and 5). In the first place, a NL method of 36/38 Da, corresponding to the NL of HCl, was performed (Table 1, method 4). Only three metabolites (S1a, S2a and S4b, Table 2) were observed in the TIC of the post-administration samples. In order to verify the presence of other sulfate metabolites, XIC chromatograms were evaluated and twelve additional peaks were detected. Only metabolite S2b was not observed when using this analytical strategy. The simultaneous use of these two NL scan acquisitions and the perfect co-elution between them facilitated the identification of the metabolites.

In the second place, a theoretical SRM method in negative mode (Table 1, method 5) which included the ion transitions from [M-H]$^-$ of potential sulfate metabolites to m/z 97 and to [M-H-HCl]$^-$ was developed. Additionally, the same ion transitions considering ^{37}Cl isotope ([M+2-H]$^-$→[M+2-H-HCl]$^-$ and [M+2-H]$^-$→97) and the transition from the in source fragment [M-H-HCl]$^-$ to m/z 97 were also added into the method. To calculate the theoretical transitions, different metabolic pathways were considered, including oxidation, reduction, hydroxylation, dihydroxylations and combinations of them. In summary, 72 ion transitions corresponding to 18 molecular masses (representing many putative sulfate metabolites) were included into the method (Figure S-1 of supplementary material (supp. mat.)). Using the SRM method, sixteen sulfate metabolites were detected in post-administration samples.

The MM and the RTs of the detected metabolites are listed in Table 2. As an example, Figure S-2 supp. mat. compares the ion transition m/z 419→383 in a pre-administration sample and in a post-administration sample (24-48h) that allowed for the detection of 8 different monohydroxylated sulfate metabolites. The detected metabolites resulted from sulfonation of oxidized or reduced (in

one or multiple sites) metabolites of CLO with or without additional hydroxylations (Table 2). The product ion mass spectra of all detected metabolites yielded the same two main ions, the ion m/z 97 (corresponding to HSO_4^-) and the ion corresponding to the neutral loss of HCl. Figure S-3 supp. mat shows the product ion mass spectra of $[M-H]^-$ in ESI negative mode of metabolites S2a, S3b, S3g and S4b.

3.2. Optimization of a LC-MS/MS method for the monitorization of CLO sulfate metabolites

A SRM method to monitor the previously detected sulfate metabolites in urine samples was developed. The mass spectrometric behaviour of the detected sulfate metabolites was comparable and the ion transitions to monitor each metabolite were selected based on signal intensity and selectivity. Having studied the product ion mass spectra, signal intensity was evaluated for five different ion transitions of each metabolite: $[M-H]^-\rightarrow[M-H-HCl]^-$, $[M-H]^-\rightarrow97$, $[M+2-H]^-\rightarrow[M+2-H-HCl]^-$, $[M+2-H]^-\rightarrow97$ and $[M-H-HCl]^-\rightarrow97$. Excretion study samples were analysed to optimize the ion transitions for each metabolite. CV and CE were optimized to obtain maximum signal for each ion transition. In most cases, the ion transitions from the in source fragments to the product ion m/z 97 ($[M-H-HCl]^-\rightarrow97$) yielded the highest signal. However these transitions were greatly interfered by endogenous peaks. Slightly lower signal intensities were obtained with the ion transitions $[M-H]^-\rightarrow[M-H-HCl]^-$, however those transitions gave the highest signal-to-noise ratios and total absence of interferent peaks at the analytes RTs. The signal intensities obtained for $[M-H]^-\rightarrow97$ were 2-3 times less intense. As expected, the 33% of the signal intensity was obtained for the transitions considering ^{37}Cl with respect to the ones from the most abundant isotope (^{35}Cl). Figure S-4 supp. mat. shows the results of the five ion transitions for metabolite S1a obtained in a pre-administration and post-administration sample (day 15). For the final SRM method, the ion transitions $[M-H]^-\rightarrow[M-H-HCl]^-$ and $[M-H]^-\rightarrow97$ 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.

3.3. Excretion profiles of sulfate metabolites in Caucasian population

The developed and optimized SRM method was applied to pre- and post-administration samples from four different Caucasian individuals. Detection times of the different sulfate metabolites are detailed in Table 2. Metabolites could be detected from a few hours up to sixteen days, except for metabolite S1a which showed the longest retrospectivity. This sulfate metabolite was detected up to the last collected sample in all studies (A: 10 days, B: 48 hours, C: 31 days and D: 31 days). Apart from S1a, four other metabolites (S2a, S3b, S3g, S4b) were detected for more than 10 days after administration. Figure 3 shows the SRM chromatograms for these five most abundant sulfate metabolites of a pre-administration sample and a post-administration sample (day 5) corresponding to excretion study C.

For comparison purposes, excretion study samples were also analysed by using the commonly employed GC-MS/MS screening methods (Table 1, method 7), after enzymatic hydrolysis and derivatization [20,23]. CLO (RT= 17.4 min) and four metabolites: M1, 4-chloro-androst-4-en-3α-ol-17-one (RT= 13.9 min); M2, 4-chloro-5α-androst-3α-ol-17-one (RT= 14.5 min); M3, 4-chloro-5β-androst-3α-ol-17-one (RT= 14.9 min); and M4: 4ξ-chloro-5ξ-androst-3α,16ξ-diol-17-one (RT= 18.1 min) excreted in the glucuronide fraction were monitored. The detection times of the metabolites are detailed in Figure 4. The longest detection times were obtained for M3 that was detected in all samples of studies A (10 days) and B (up to 36-48h), and was detected up to 20 and 17 days in studies C and D, respectively. M2 and M4 showed a detection window between 7 to 17 days depending on the volunteer. Finally, M1 was detected up to 5-6 days and CLO no longer than 72h. Figure 4 summarizes the detection times obtained for the most abundant sulfate metabolites (S1a, S2a, S4b) and glucuronide metabolites (after hydrolysis, derivatization and GC-MS/MS analysis). In

summary, the sulfate metabolite S1a increases the time in which CLO administration is detectable with respect to commonly monitored metabolites.

3.4. Characterisation of the sulfate metabolite S1a

To identify the structure of the sulfate metabolite S1a, the product ion mass spectrum was evaluated (Figure 2). The [M-H]$^-$ was 403 corresponding to a molecular mass (MM) of 404 Da. Thus, the MM of the phase I metabolite was 324 Da, which either corresponds to the structure of CLO with a reduction or with an oxidation and two reductions. In Figure 2, product ion spectra of m/z 403 ([M-H]$^-$) and m/z 405 ([M+2-H]$^-$) are shown at different collision energies. Product ion spectrum of [M-H]$^-$ exhibited ions at m/z 97 and at m/z 367, corresponding to a neutral loss of HCl (Figure 2, A). The same product ions were obtained for [M+2-H]$^-$ (m/z 405) (Figure 2, B).

The information obtained from the product ion mass spectrum of S1a was not sufficient for structure identification and, therefore, a different approach was applied. The LC fraction of the peak corresponding to the sulfate metabolite S1a was isolated and a solvolysis procedure was applied in order to release the corresponding phase I metabolite (metabolite X). The fraction was analysed by GC-MS in scan mode, after forming the corresponding enol-TMS derivative. Figure 5 shows the extracted ion chromatograms of a blank urine and a sample collected after CLO administration which were subjected to β-glucuronidase hydrolysis, liquid-liquid extraction and derivatization (Figure 5, A and B) and, the post-administration sample after direct extraction of sulfate metabolites, LC fraction collection, solvolysis and derivatization (Figure 5, C). The extracted ions 468.4 and 453.4 correspond to metabolite X and, also, to two of the CLO commonly detected metabolites excreted as glucuronides: M2 and M3. Metabolite X showed a peak at 16.4 min (Figure 5, C), while metabolites M2 and M3 showed peaks at 14.5 min and 14.9 min, respectively (Figure 5, B). None of these peaks was present in the blank urine sample.

These results indicated that metabolite X, detected after solvolysis, did not correspond to either M2 or M3. The electron ionization mass spectrum of the bis-O-TMS derivative (Figure 6) showed very similar ions and relative abundances of the ions for all three metabolites: M2 (Figure 6, A), M3 (Figure 6, B) and X (Figure 6, C). The presence of the characteristic ion at m/z 169, corresponding to the D-ring containing a 17-keto group, indicates an oxidation of the C17 hydroxyl group of CLO and suggests two reductions in the A-ring. Therefore, all these data indicates that metabolite X is an isomer of metabolites M2 and M3 with different configuration in the A-ring. This is in agreement with previous results that shown that 3α -metabolites are mainly excreted as glucuronides and 3β-metabolites as sulfates [3,21]. Because of the low prevalence of 5β,3β isomers after reduction of the A ring [3,21], the most feasible configuration for metabolite X is 4ξ-chloro-5α-androst-3β-ol-17-one and, therefore, metabolite S1a is 3β-sulfate. Another evidence of the 3β-configuration of S1a is the difference in RT of the TMS derivatives of metabolites M3 and X (*c.a.* 1,5 min), which is the same as the observed for the TMS derivatives of androsterone (RT= 10.5 min) and epiandrosterone (RT= 12.0 min) which only differ in their α/β configuration in position 3 as metabolites M3 and X. The proposed structure for metabolite S1a is depicted in Figure 1.

4. Discussion

Phase II metabolic reactions of AAS have been traditionally studied by using specific hydrolysis to obtain unconjugated metabolites that were subsequently determined by GC-MS or LC-MS [3,5,22,24]. In the recent years, LC-MS/MS technology has allowed the direct detection of phase II metabolites, including glucuronides and sulfates. Although sulfate conjugates have not been systematically studied, some recent studies showed their importance to improve the retrospectivity for the detection of AAS misuse [8-12,15]. Moreover, the relevance of sulfate conjugates on CLO metabolism was recently demonstrated for Chinese population [15]. However, inter-ethnic variations of the metabolizing enzymes can cause large differences in the excretion of AAS conjugates [16,25]. In the present study, the sulfate fraction of CLO metabolism was evaluated in Caucasian population in order to further understand the metabolism of this doping agent.

Different strategies were applied to directly detect sulfate metabolites of CLO. They were based on both, the already known mass spectrometric behaviour of sulfate metabolites and a new finding obtained during this study. Sulfate metabolites have common ions and losses that are useful for the detection of unknowns [8,22]. Thus, a PI method of m/z 97 and a NL method of 80 Da (corresponding to HSO_4^- and the loss of the sulphur trioxide group, respectively) were employed. The PI of m/z 97 allowed the detection of 16 sulfate metabolites, when extracting chromatograms of the expected metabolites, whereas NL of 80 Da did not show any peaks meaning that these metabolites do not ionize in positive mode and therefore lack a 3-keto function. On the other hand, the study of the mass spectrometric behaviour of CLO and its metabolites available as standards, revealed the presence of the ions m/z 131 and m/z 143 that were used to develop another PI method. These ions are in accordance with those detected for testosterone analogues (m/z 109 and m/z 97) that only differ with CLO in the Cl [26]. It is important to note that this approach was limited to CLO metabolites with unmodified A-ring and having a 3-keto function. Results did not show any peaks

indicating that detected CLO sulfate metabolites were reduced in 3 or had other structural modifications in the A ring.

Subsequently, an in-depth study of the CID spectra of the [M-H]$^-$ ions showed a NL of HCl common to all detected metabolites that had not been previously described. The NL was also applied to excretion samples. Finally, a SRM method with theoretical transitions of potential sulfate metabolites was also applied, using as precursor ions the [M-H]$^-$ of the potential sulfate metabolites, and as product ions the ions at m/z 97 and at [M-H-HCl]$^-$. Transition [M-H]$^-$ to [M-H-HCl]$^-$ demonstrated to be the option of choice since it was found to be more sensitive and selective than transition [M-H]$^-$ to m/z 97 and less interfered that transition [M-H-HCl]$^-$ to m/z 97. Due to its inherent sensitivity, the use of the theoretical SRM method allowed for the determination of the largest number of metabolites (sixteen).

Further, our research was focused on the optimization of an SRM method to analyse excretion study samples from four Caucasian individuals, in order to determine the most retrospective sulfate metabolite. The results obtained after the analysis of the excretion study samples revealed metabolite S1a as the most retrospective marker (Table 2, Figure 4). Metabolite S1a was detected up to the last collected sample in all studies, so longer collection periods would be necessary to evaluate its detection time after CLO administration. S1a showed to be the long-term metabolite and its signal intensities in urine were 20 times higher compared to the second most intense metabolite (S2a). Sulfate metabolites S2a, S3b, S3g and S4b were detected up to 10 days after CLO administration.

The identification work was only focused on metabolite S1a. Few structural information was obtained from the product ion mass spectra of [M-H]$^-$ since only two product ions, corresponding to the hydrogen sulfate anion and the ion resulting from the neutral loss of HCl, were observed. Despite not being sufficient data for the metabolite characterization, the existence of two ion transitions

ensures the possibility to perform confirmation analyses according to WADA regulations [27]. Finally, the analytical data obtained from metabolite X (phase I metabolite released from S1a after a solvolysis procedure) using GC-MS analysis allowed the identification of S1a as 4ξ-chloro-5α-androst-3β-ol-17-one 3β-sulfate (Figure 1). The phase I metabolite, metabolite X, was not detected after analysis of the sample using β-glucuronidase hydrolysis indicating that it is only excreted as conjugated with sulfate. Nevertheless, confirmation of S1a structure would require synthesis of the compound and comparison of analytical data with that obtained from the compound detected in urines.

The structure of a CLO phase I metabolite excreted as a sulfate was already suggested in early metabolic studies using hydrolysis and GC-MS analysis [3]. Also, a sulfate metabolite with the same MM was recently reported for Chinese population and it was detected up to 25 days after administration to one Chinese volunteer [15]. The structure proposed for that sulfate metabolite was 4ξ-chloro-5ξ-androst-3ξ-ol-17-one 3ξ-sulfate, based only on mass spectrometric data obtained after LC-QTOF analysis. It would be of interest to verify if the structure of the metabolite detected in the Chinese volunteer is the same as the one detected in Caucasian population in our study or if they are isomers. It is important to notice that the Chinese volunteer was administered with 40 mg of CLO whereas volunteers C and D were only administered 10 mg. The longest detection time obtained in our work could be due to inter-ethnic or inter-individual variability or due to the use of a more sensitive analytical method (sample preparation and instrumental analysis) in our study.

Up to now, the currently employed method for the detection of CLO misuse is based on the hydrolysis of glucuronoconjugated metabolites and subsequent analysis by GC-MS/MS [20,23]. M1, M2 and M3 are the current markers used to detect CLO administration although CLO and M4 can be also detected using that methodology. M2, M3 and M4 have detection times close to 10 days and M3

has the longest retrospectivity. In the recently published work about CLO metabolism in Chinese population [15], the direct analysis of glucuronide metabolites using LC-MS/MS was also performed, and the detection times obtained were much lower than those obtained when using the traditionally employed screening methods by GC-MS/MS. These results could indicate a different metabolism regarding glucuronidation between Asian and Caucasian population [16] or a low sensitivity of the LC-MS/MS analysis for steroid glucuronides. The lower sensitivity for steroid glucuronides using direct LC-MS/MS approaches compared to the indirect GC-MS/MS analyses of the aglycones after enol-TMS derivatization, especially for glucuronides of totally A-ring reduced steroid phase I metabolites, was already observed for methyltestosterone glucuronide metabolites in a previous study of our group [14].

The results obtained in our study indicate that the best detection window to monitor CLO administration is obtained using the sulfate metabolite S1a and, therefore, it is the best marker to be included into the recently developed screening methods for AAS consisting on the direct detection of phase II metabolites [13,14].

In summary, the sulfate metabolite S1a is a long-term metabolite in Caucasian population. These results might be in accordance with those obtained for Chinese population. Therefore, inclusion of S1a into screening methods is advisable for the doping control analysis.

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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 +	131/143	25	35	350-500
2	PI	ESI -	97	30	35	350-500

Method	**Scan mode**	**Ionization mode**	**NL (Da)**	**CV (V)**	**CE (eV)**	**Range (m/z)**
3	NL	ESI +	80	30	10	350-500
4	NL	ESI -	36/38	35	20	350-500

Method	**Scan mode**	**Metabolite**	**PI (m/z)**	**DI (m/z)**	**CV (V)**	**CE (eV)**
5	SRM	Putative sulfate metabolite	[M-H]⁻	[M-H-HCl]⁻	30	20
			[M-H]⁻	97	30	35
			[M-H-HCl]⁻	97	55	35
			[M+2-H]⁻	[M+2-H-HCl]⁻	30	20
			[M+2-H]⁻	97	30	35

TARGETED DETECTION						
Method	**Scan mode**	**Metabolite**	**PI (m/z)**	**DI (m/z)**	**CV (V)**	**CE (eV)**
6	SRM	S1	403	367	30	20
			403	97	30	35
		S2	405	369	30	20
			405	97	30	35
		S3	419	383	30	20
			419	97	30	35
		S4	421	385	30	20
			421	97	30	35
		S5	435	399	30	20
			435	97	30	35

Method	**Scan mode**	**Metabolite**	**PI (m/z)**	**DI (m/z)**		**CE (eV)**
7	SRM	CLO	466	431		23
			466	335		23
		M1	466	451		5
			431	181		15
		M2	468	453		10
			453	363		15
		M3	468	453		10
			453	237		20
		M4	556	541		20
			558	543		20

Table 2. Sulfate metabolites of CLO detected in post-administration samples, metabolic pathway, [M-H]⁻ ion, retention time (RT) and detection times in the four evaluated excretion studies (CS: CLO sulfate; red: reduction; ox: oxidation; OH: hydroxylation).

Metabolite	Metabolic pathway	[M-H]⁻ (m/z)	RT (min)	Detection times in excretion study urines			
				Study A	Study B	Study C	Study D
S1a	CS+2red+1ox or CS+1red	403	16.3	Day 10*	36-48h*	Day 31*	Day 31*
S1b			18.1	Day 10*	36-48h*	Day 9	Day 9
S1c			19.4	Day 7	36-48h*	Day 7	96-108h
S2a	CS+2red	405	13.0	Day 10*	36-48h*	Day 13	Day 12
S2b			21.0	Day 7	36-48h*	Day 7	84-96h
S3a	CS+1ox+2red+1OH or CS+1red+1OH	419	2.4	Day 7	36-48h*	24-36h	Day 8
S3b			3.3	Day 5	36-48h*	Day 11	Day 14
S3c			4.1	Day 10*	36-48h*	84-96h	Day 9
S3d			5.9	Day 7	36-48h*	12-24h	24-36h
S3e			6.4	Day 5	36-48h*	Day 9	Day 6
S3f			9.2	Day 5	36-48h*	Minor	minor
S3g			10.1	Day 10*	36-48h*	Day 11	Day 9
S3h			11.1	Day 7	36-48h*	Day 6	60-72h
S4a	CS+2red+1OH	421	2.9	Day 10	36-48h*	minor	96-108h
S4b			3.6	Day 10*	36-48h*	Day 11	Day 16
S5a	CS+1ox+2red+2OH or CS+1red+2OH	435	2.8	Day 10*	36-48h*	72-84h	72-84h

* Last collected sample

FIGURE LEGENDS

Figure 1. Structure of clostebol (CLO) and proposed structure of sulfate metabolite S1a.
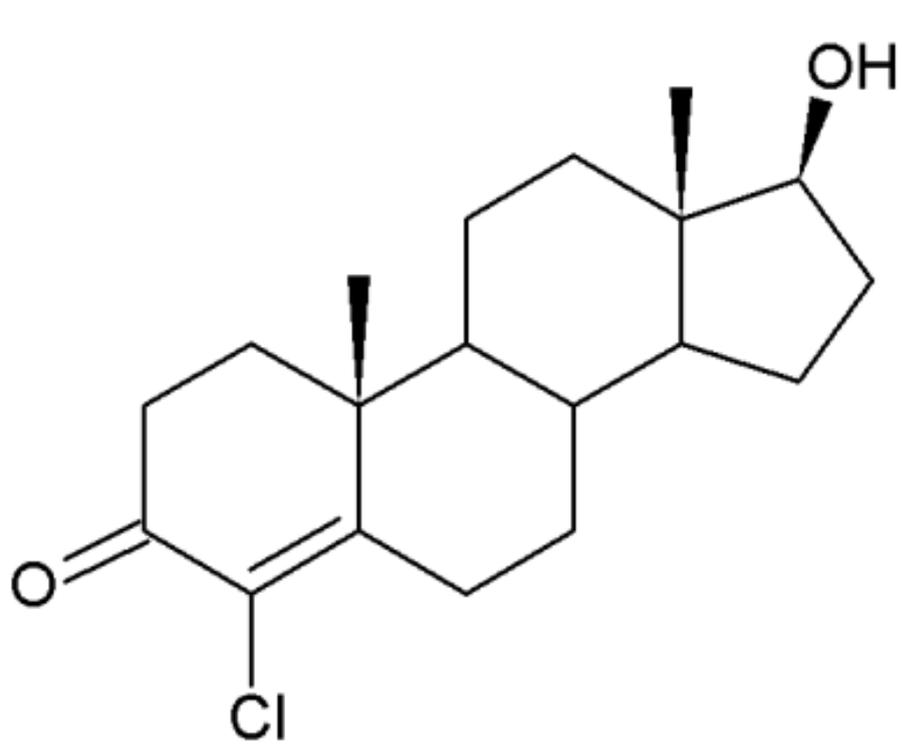
Figure 2. Product ion mass spectra of [M-H]$^-$ and [M+2-H]$^-$ of metabolite S1a in ESI negative mode at three different collision energies (CV=30V; CE= 15, 25, 35eV).

Figure 3. LC-MS/MS results: SRM chromatograms (transitions corresponding to metabolites S1a, S2a, S3b, S3g, and S4b) of a pre-administration sample and a post-administration sample (day 5) corresponding to excretion study C.
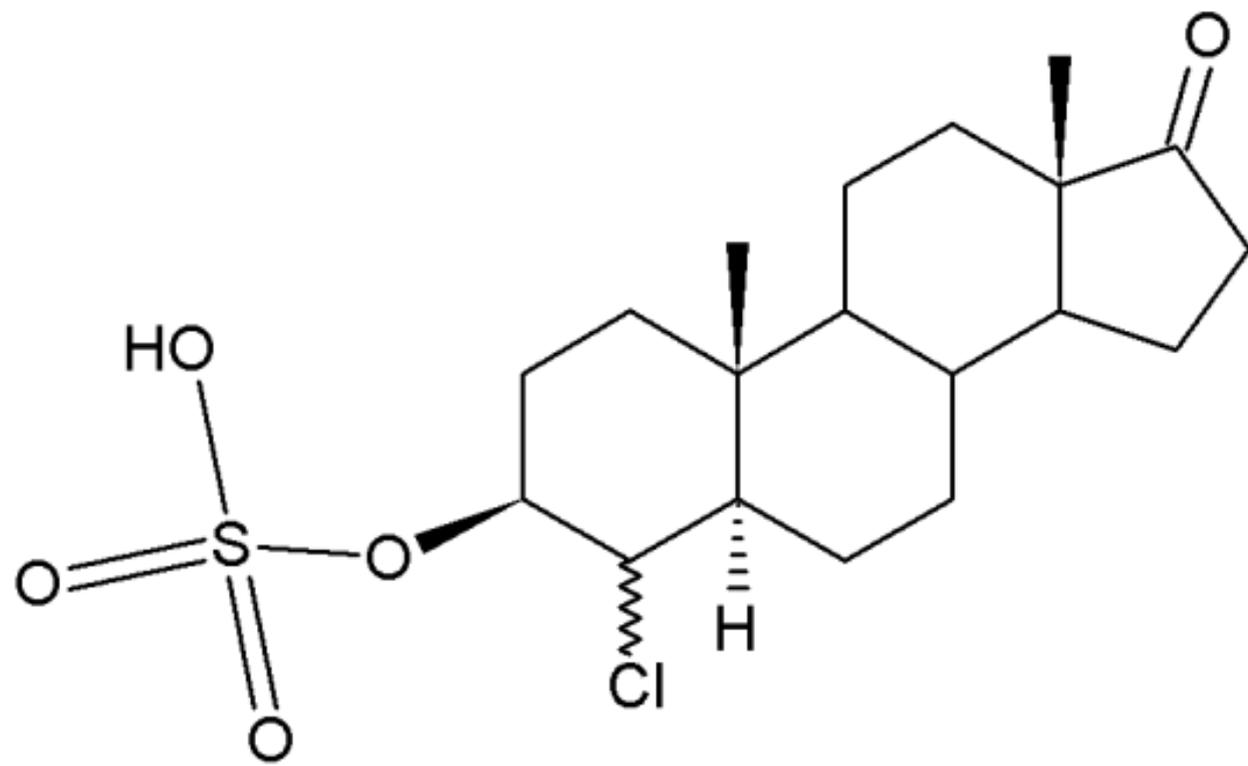
Figure 4. Detection times of the different metabolites of CLO obtained for the four excretion studies using GC-MS/MS analysis after enzymatic hydrolysis and derivatization (CLO and metabolites M1 to M4), or using direct LC-MS/MS analysis of sulfate metabolites (metabolites S1a, S2a and S4b).

Figure 5. Identification by GC-MS of metabolite X released after solvolysis of S1a. Extracted ion chromatograms (453 (black), 468 (red)) of the bis-TMS derivative of metabolites M2, M3 and metabolite X of: A, blank urine; B, sample collected after CLO administration after β-glucuronidase hydrolysis, liquid-liquid extraction and derivatization; and C, sample collected after CLO administration after direct extraction of sulfate metabolites, LC fraction collection, solvolysis and derivatization.

Figure 6. Structure and electron ionization mass spectra of the bis-O-TMS derivative of metabolite M2 (A) and M3 (B), and metabolite X released after solvolysis of S1a (C).

CLO   S1a

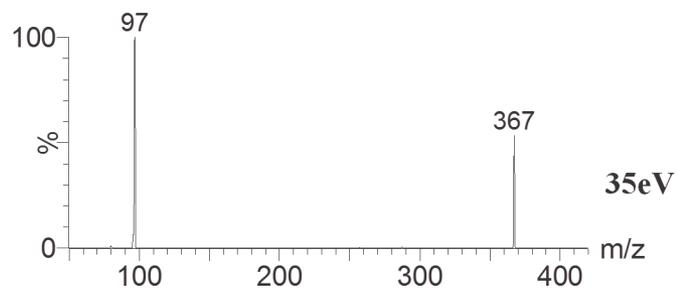**A)** Product ion scan m/z 403 (^{35}Cl)

B) Product ion scan m/z 405 (^{37}Cl)

	S1a 403→367	**S2a** 405→369	**S3b** 419→383	**S3g** 419→383	**S4b** 421→385
Pre-adm	2.37e3 Area	646 Area	698 Area	812 Area	777 Area
Day 5	6.32e5 Area	7.72e3 Area	2.04e3 Area	1.78e3 Area	5.62e3 Area

A)
x10 5 + EIC(453) Scan 0201002.D

B)
x10 5 + EIC(453) Scan C063U015.D

M3
14,9

M2
14,5

C)
x10 4 + EIC(453) Scan Fracció 4_S1b_1,2aliq.D

Metabolite X
16,4

Counts vs. Acquisition Time (min)

A) + Scan (14.580 min) C063U015.D Subtract

B) + Scan (14.938 min) C063U015.D Subtract

C) + Scan (16.412 min) Fracció 4_S1b_1,2aliq.D Subtract