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Hair analysis can provide additional information in doping and forensic cases involving clostebol

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Hair analysis can provide additional information in doping and forensic cases involving clostebol

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Keywords:	doping, clostebol, hair analysis, Trofodermin, 4-chlorotestosterone
Abstract:	Clostebol is a synthetic anabolic androgenic steroid, with potential use as a performance-enhancing drug if taken for long periods in order to produce the desired effect. Recently, the use of medications containing clostebol acetate has led to the suspension of several athletes in various sports. Previous studies have showed that urine can result positive in case of single intake of a banned substance, including unintentional consumption of steroids. In this context, hair test can contribute to exculpation of athletes by demonstrating alternative administration or contamination. The development and validation of a UHPLC-MS/MS method to detect clostebol and clostebol acetate in hair is hereby presented. Some real cases of athletes sanctioned for clostebol use, in which we analyzed hair samples to follow-up investigations of doping control laboratories and obtain useful elements to understand the origin of clostebol intakes, and two forensic cases of anabolic drugs abuse, are also presented and discussed. In real head and body hair samples, clostebol acetate could be detected in the low pg/mg range. As is typical of hair analysis, the interpretation of the quantitative findings may be challenging, and even more in sports owing to the lack of systematic studies. However, the results can be used to produce evidence contrary to any ruling issued against the athletes by the appropriate sports body, so to possibly obtain a diminished sanction. Because the sport authorities do not make a distinction among circumstances or means of administration of anabolic compounds, athletes should be warned not to use clostebol-containing medications.

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Hair analysis can provide additional information in doping and forensic cases involving clostebol

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Keywords: doping; Clostebol; hair analysis; Trofodermin; 4-chlorotestosterone

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Abstract

Clostebol is a synthetic anabolic androgenic steroid, with potential use as a performance-enhancing drug if taken for long periods in order to produce the desired effect. Recently, the use of medications containing clostebol acetate has led to the suspension of several athletes in various sports. Previous studies have showed that urine can result positive in case of single intake of a banned substance, including unintentional consumption of steroids. In this context, hair test can contribute to exculpation of athletes by demonstrating alternative administration or contamination. The development and validation of a UHPLC-MS/MS method to detect clostebol and clostebol acetate in hair is hereby presented. Some real cases of athletes sanctioned for clostebol use, in which we analyzed hair samples to follow-up investigations of doping control laboratories and obtain useful elements to understand the origin of clostebol intakes, and two forensic cases of anabolic drugs abuse, are also presented and discussed. In real head and body hair samples, clostebol acetate could be detected in the low pg/mg range. As is typical of hair analysis, the interpretation of the quantitative findings may be challenging, and even more in sports owing to the lack of systematic studies. However, the results can be used to produce evidence contrary to any ruling issued against the athletes by the appropriate sports body, so to possibly obtain a diminished sanction. Because the sport authorities do not make a distinction among circumstances or means of administration of anabolic compounds, athletes should be warned not to use clostebol-containing medications.

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Introduction

Clostebol, also known as 4-chlorotestosterone, is a weak synthetic anabolic androgenic steroid, with potential use as a performance-enhancing drug. For this reason, it is currently banned by the World Anti-Doping Agency[1]. For doping purposes and remarkable anabolic effects, clostebol is typically used as an ester, such as clostebol acetate, caproate, and propionate. Its intake usually occurs either by oral ingestion (as a tablet) or by intra-muscular injection, the latter generating high clostebol concentrations in biological samples. Even when clostebol is consumed in micro-doses (i.e., generating low levels of its main metabolite in urine), its intake should be maintained for some weeks in order to produce the desired effect. As clostebol acetate, it is also contained in balm and cream products, the most common being Trofodermin®. In recent years, the alleged use of clostebol led to the suspension of a number of athletes in various sports [2]. Italy is one of the countries with the highest anti-doping rule violations involving this substance. This is not surprising, since Italy is one of few countries (another one is Brazil) who sell clostebol-containing medicines for dermatologic and gynecologic treatments. For example, the US Food and Drug Administration does not allow the inclusion of anabolic agents in any prescription [3]. The same occurs in France and in most European countries. Incidental clostebol contamination in athletes after sexual intercourse [3] or from contaminated food [4,5] was also demonstrated in a few circumstances.

Several cases of Adverse Analytical Finding (AAF) for banned doping agents detected in urine required the use of hair analysis to provide complementary information and understand the origin of the positive finding [6-8]. As a matter of fact, the major disadvantage of urinalysis is that it provides only short term information of the individual's drug consumption and does not ascertain sustained drug abuse [9]. Thus, urine analysis is not particularly effective at identifying the athletes who partake in long term steroids use during the training periods and cease to take them prior of competition, resulting in a drug-free period sufficient to give negative results [10]. In an opposite scenario, urine analysis may yield a positive result when a single intake of a banned substance occurred, including unintentional steroids consumption [11,12]. In the latter case, the hair specimen would turn out negative. Although hair testing is not suitable to overrule a positive urine finding, it can significantly contribute to the exculpation of innocent athletes by demonstrating alternative administration pathways or contamination risks [13]. Recently, this role of hair testing has been demonstrated in a case where hair analysis allowed the discrimination between the mycotoxin zearalenone and its urinary metabolite zeranol, which is banned for its anabolic properties [14]. In the present study, we developed a UHPLC-MS/MS method to detect clostebol and clostebol acetate in hair and applied this method to three real cases of athletes sanctioned for clostebol use, in order to

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3 follow-up investigations of doping control laboratories and obtain useful elements to understand the
4 origin of these positive findings, and two forensic cases in which some anabolic drugs abuse was to
5 be assessed.
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10 EXPERIMENTAL
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12 Materials
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15 Chemical reagents, including methanol and acetonitrile (ACN), were purchased from Sigma-
16 Aldrich (Milan, Italy). Clostebol and testosterone-d3 were purchased from Steraloids (USA).
17 Clostebol acetate was purchased from Sigma-Aldrich (Milan, Italy). Ultrapure water was obtained
18 by a Milli-Q Millipore system (Bedford, MA, U.S.A.). A Precellys 24 Tubes Homogenizer (Bertin
19 Pharma, Montigny-Le-Bretonneux, France) equipped with six 2.8 mm metal beads was used for
20 hair milling. Stock standard solutions of analytes and internal standard (IS), were prepared in
21 methanol at a concentration of 1 mg/mL and stored at −20°C in dark vials. Working methanolic
22 solutions containing all the analytes at different concentrations were prepared by mixing the stock
23 solutions at the proper dilution. The working solutions were used to spike negative hair samples at
24 various levels.
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32 Instrumental conditions
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34 All analyses were performed on a Shimadzu Nexera 30 UHPLC-system (Shimadzu, Duisburg,
35 Germany) interfaced to a Sciex API 5500 triple quadrupole mass spectrometer (Sciex, Darmstadt,
36 Germany) with an electrospray Turbo Ion source operating in the positive ion mode (ESI+). A
37 Waters BEH C18 column 100 × 2.1 mm i.d. × 1.8 μm, protected by a C18 guard column, was used
38 for the separation of analytes. The column oven was maintained at +45 °C, and the elution solvents
39 used were water/ammonium formate 5 mM (solvent A) and acetonitrile/ammonium formate 5 mM
40 (solvent B). The mobile phase eluted under the following conditions (A/B; v/v): initial 90:10 ratio
41 for 0.5 min, then linear gradient to 10:90 in 7 min; final isocratic condition at 90% B for 0.5 min.
42 The flow rate was 0.5 mL/min. The MS system was operated in the selected reaction monitoring
43 mode (SRM). In order to establish appropriate SRM conditions, each analyte was individually
44 infused into the ESI capillary, while the declustering potential (DP) and the entrance potential (EP)
45 were adjusted to maximize the intensity of the $[M–H]^+$ species. The collision offset voltage (CE)
46 was adjusted to preserve approximately 10 % of the precursor ion, and the cell exit potentials (CXP)
47 were also optimized. Each SRM transition was maintained during a time window of ±10.0 s around
48 the expected retention time of the corresponding analyte, and the SRM target scan time (i.e.,sum of
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dwell times for each SRM cycle) was 0.30 s, including pause times of 5 ms between consecutive SRM transitions. The best results were obtained using a source block temperature of +600 °C and an ion-spray voltage of 3800 V. Both Q1 and Q3 were operated at unit mass resolution. The gas settings were as follows: curtain gas 35.0 psi, collision gas 8.0 psi, ion source gas GS1 45.0 psi, and ion source gas GS2 55.0 psi. The Analyst 1.5.2 (Sciex) software was used for data processing. All analytes and IS, their corresponding retention time, SRM transitions, and potentials are presented in Table 1.

Sample preparation

About 50 mg of hair was twice-washed with dichloromethane and methanol (2 mL, vortex mixed for 3 min). After complete removal of the solvent wash, the hair was dried at room temperature by a gentle nitrogen flow and subsequently pulverized using a metal beads mill. The samples were then fortified with 5 µL of testosterone-d3 at 250 pg/µl, yielding a final concentration of 25 pg/mg. Sample extraction was carried out by addition of 1 mL of methanol, vortex shaking for 0.5 min, centrifugation at 4000 rpm for 1 min, to ensure the complete immersion of the matrix into the solvent, and final incubation at 55°C for 15 h. Lastly, the organic phase was collected and evaporated to dryness under a gentle stream of nitrogen and mild heating (70°C) using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was dissolved in a 50 µL methanol/ACN 1:1 solution, transferred into a vial and centrifuged at 4000 rpm for 10 min. A 5 µL aliquot was injected into the UHPLC–MS/MS system.

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Validation

The analytical method was validated in accordance with the criteria and recommendations of international standard and international guidelines [15]. The following parameters were investigated: specificity, selectivity, linearity range, detection and quantification limits (LOD and LOQ), intra-assay and inter-assay precision and accuracy. The calibration process was conducted with an optimized procedure, requiring the preparation of three replicates of the calibration curves for the two analytes on three different days for a total of nine calibration curves for each analyte. The data from each specific calibration curve were quantified using a calibration curve obtained on a different day, allowing to manage each set of data as independent. Therefore, 9 samples (from 9 different batches) per each calibration level were employed to estimate the previously cited validation parameters. Carry-over and matrix effect were also investigated.

Specificity

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A pool of five blank hair samples obtained from different healthy volunteers was prepared without spiking and analyzed as described above. The occurrence of possible interferences from endogenous substances was tested by monitoring the SRM chromatograms characteristic for each investigated compound at the expected retention time interval. The S/N was measured on the less intense mass transition at the expected analyte retention time. The noise was measured from the end of the peak till ±0.05 min after it for each analyte. A S/N<3 was considered satisfactory in order to verify the method's specificity.

Selectivity

The repeatability of relative peak intensities for the transitions of each analyte was determined on five replicates of the pooled blank hair sample fortified at two concentration levels (10 and 100 pg/mg). To assess selectivity, one qualifying transition was monitored, in addition to the primary transition.

Linearity, LOD and LOQ

The linear calibration model was checked by analyzing blank hair samples spiked with the working solution at five concentration levels (1, 10, 25, 50, 100 pg/mg). The calibration was completed by internal standardization. The linear calibration parameters were evaluated using the least squares regression method; several significance tests were performed to evaluate linearity, including lack-of-fit test, analysis of variance (ANOVA), Mandel's test, and homo- vs. heteroscedasticity tests (see Table 2a). Determination coefficient (R^2), adjusted determination coefficient (Adj R^2), relative standard deviation of the slope, normality of the standardized residuals, and deviation from back-calculated concentrations were also evaluated. The limit of detection (LOD) and the limit of quantitation were estimated with the Hubaux-Vos' approach [16], which consider the uncertainty in the signals and concentrations associated with the calibration set. In-house developed spreadsheets, together with the routines developed by Desharnais *et al.* [17], were employed to evaluate these parameters and perform their relative significance tests.

Precision and accuracy

For all analytes, intra- and inter-day precision (expressed as percent variation coefficient, CV%) and accuracy (expressed as bias %) were evaluated at three concentration levels. The samples prepared for the evaluation of linearity, LOD and LOQ parameters were used, thus consisting of nine replicates (prepared and analyzed in three different days) of blank hair samples spiked with the standard solutions at the lowest calibration point (close to LOQ values), at intermediate calibration

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level, and the highest calibration point, i.e. 1, 25 and 100 pg/mg concentrations. Intra-day and inter-day precision parameters were considered satisfactory when CV% values turned below 15%. Satisfactory accuracy (in terms of bias %) was achieved when the experimentally determined average concentration lied within ±15% from the expected value.

Carry-over

The background chromatographic profiles for each analyte were monitored during the analysis of a blank hair sample injected for five times after the chromatographic run of a spiked blank hair sample containing all the analytes at 250 pg/mg concentration. To assure the absence of any carry-over, the same criteria adopted to verify the specificity requirements had to be fulfilled.

Matrix effect

The matrix effect was calculated relatively to the ISTD, by comparing the peak area ratio between analyte and ISTD obtained from spiked hair samples, with the corresponding ratio obtained from a pure methanol solution, at the same concentrations. In this case, the matrix effect is expected to be partly compensated by a well-matched internal standard, i.e. the isotopically-marked analyte, whenever possible, or the one having the closest RT to the analyte, so as to undergo similar interference from the matrix. The matrix effect was calculated as the mean value obtained from five different hair sources. The percent difference represented either matrix suppression (values below 100%) or matrix enhancement (values above 100%) [18].

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RESULTS

Validation

The optimized UHPLC-MS/MS method allowed the simultaneous determination of the two analytes plus the IS. The whole chromatographic run, comprehensive of the time required for column re-equilibration, was completed in 9.0 min. Three SRM transitions were selected for each analyte. Validation results are presented in Table 2a and 2b.

Specificity and Selectivity

SRM chromatograms from negative hair samples showed no interfering signals at the retention time where the analytes were expected to elute. Thus, all specificity tests proved successful. Variations of relative qualifier ion intensities did not exceed ±20% with respect to the corresponding control and were considered acceptable, as well the retention times.

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3 Linearity, LOD and LOQ
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5 The trend of the variance associated to the tested calibration points (i.e. homoscedasticity vs.
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7 heteroscedasticity) was evaluated. A weighting factor equal to ($1/x^2$), where x represents the
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9 concentration of the calibration point, was adopted for both clostebol and clostebol acetate because
10 the data points distribution turned heteroscedastic. The Mandel's test suggested a linear regression
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12 model for both the target analytes. Then, both lack-of-fit and ANOVA tests turned successful for
13 clostebol, while only lack-of-fit test was not passed for clostebol acetate. Table 2a reports a
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15 summary of the observed results, including the Adj R^2 values of the regression models. LOD and
16 LOQ values estimated by Hubaux-Vos' methodology are reported, too. In particular, LOD values
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18 were0.3 pg/mg for clostebol and 1.1 pg/mg for clostebol acetate, while LOQ values lied at 0.6
19 pg/mg and 2.1 pg/mg, respectively. The calculated LODs were experimentally confirmed with one
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21 blank hair sample spiked at the estimated LODs concentrations.
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24 Precision and accuracy
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26 Intra-day precision, inter-day precision and accuracy parameter results are reported in Table 2b.
27 They showed satisfactory intra-day repeatability, as the percent variation coefficient (CV%) turned
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29 lower than 15% for all the spiked analytes at low, medium and high concentration (i.e. 1, 25 and
30 100 pg/mg), with the exception of the lowest calibration point of clostebol that showed a CV%
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32 equal to 20%, but a consistent average value of 0.9 pg/mg and a standard deviation of 0.1 pg/mg.
33 Also inter-day repeatability results proved fully satisfactory, as CV% values were within 15% or
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35 lower for all the spiked analytes at both low, medium and high concentrations, with once again the
36 exception of the lowest calibration point of clostebol that showed a CV% equal to 30%. Finally, the
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38 accuracy parameter turned optimal, as the percent bias were within few percent in almost all cases,
39 with maximum experimental errors of -9% for clostebol and -4% for clostebol acetate.
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46 Carry-over
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48 The background chromatographic profiles of the main transitions for each analyte, monitored
49 during the analysis of blank hair extracts injected after samples spiked at the highest analytes
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51 concentration, did not show the presence of any significant signal (i.e., the S/N value was always
52 <3) at the retention times expected for both analytes.
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55 Matrix effect
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The effect of the real hair matrix appeared to be not significant for both analytes tested. In general, the good linearity observed in the calibration plots demonstrated that the observed matrix effect is proportionally constant, i.e., does not depend on the analytes' concentrations.

REAL CASES

Case 1

A professional boxer, male, 33 years old, was found positive in urine after an *in-competition* control (clostebol metabolite: 1 ng/mL). He claimed to have applied an ointment containing clostebol acetate (Trofodermin®, 500 mg/100 gr) to his wife for 4-5 days, one time per day, in order to heal her infected shoulder tattoo. He also claimed he did not know that the cream was containing a banned substance. Last application was done one week before the antidoping control. Three months later, we collected arm hair and leg hair from the athlete, and head hair from his wife. Head hair and chest hair from the athlete were too short to cover the period of interest therefore they were not collected. All samples were black-colored. Arm and leg hair were analyzed in their total length, while the wife's' hair was segmented according to the period of interest. A full description of the collected samples is presented in Table 3.

Arm hair and leg hair collected from the athlete resulted positive to clostebol acetate (respectively 3 pg/mg and 5 pg/mg). Only one segment of wife's hair was positive (13 pg/mg). Clostebol was not detected in any sample. The chromatogram from the analysis of the leg hair sample is shown in Figure 1.

Case 2

A 45-years-old long-distance female runner was found positive after an *in-competition* urine control (clostebol metabolite 15 ng/mL). She admitted the use of Trofodermin® before the competition to medicate some foot lesion. She also claimed she did not know that the cream was containing a banned substance. A hair sample, black colored, was collected 45 days after the antidoping control and analyzed after segmentation according to the period of interest. A full description of the collected samples is presented in Table 3.

Only one hair segment resulted positive for clostebol acetate at the concentration of 21 pg/mg. Clostebol was not detected in any segment.

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Case 3

A young male diver, 18 years old, was found positive after an *in-competition* urine control (clostebol metabolite 1 ng/mL). The use of Trofodermin® or other clostebol-containing medicines was not acknowledged. Several nutritional supplements and medications were also tested for the presence of clostebol. Possible contamination from beef meat eaten in a Japanese restaurant few days before the antidoping control was also accounted for the positive result. We collected head hair and pubic hair 30 days after the antidoping control. All samples were black colored. Pubic hair was analyzed in its total length, while the head hair was segmented according to the period of interest. A full description of the collected samples is presented in Table 3.

All samples were negative for clostebol and clostebol acetate, including hair samples, nutritional supplements and medications.

Case 4

A man, aged 41 years was under a divorce procedure. The man declared discontinuation of any anabolic drug that he admitted to use previously to improve his performance in the amateur sport practice within a fitness center. A hair test was requested by the judge to discriminate the unceasing use of anabolic drugs from their discontinuation in order to decide which parent should be responsible of two children. A full description of the collected samples is presented in Table 3. Unfortunately, a segmental analysis was not feasible because of the low quantity of hair available.

The test executed on the entire 3-cm hair sample revealed the presence of clostebol acetate at 9 pg/mg.

Case 5

A man, aged 35 with aggressive behaviour, was accused to have allegedly raped a young girl. He declared the use of anabolic drugs to the judge. He also declared that the violence was caused by an enhanced libido induced by his abuse of anabolic steroids. The laboratory was requested to verify his statement. A full description of the collected samples is presented in Table 3.

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The hair sample was found positive to clostebol acetate (6 pg/mg). Other anabolic steroids, including stanozolol, methandienone and boldenone, were detected in the same hair sample (data not shown).

A full description of results is presented in Table 3.

Discussion

Synthetic anabolic steroids have attracted significant scientific attention as target compounds in hair analysis for both doping control and a variety of different forensic contexts [13]. In this study, we report for the first time the detection of clostebol acetate in hair in the pg/mg range. In hair analysis, the interpretation of the quantitative findings is typically challenging, and even more in antidoping analysis (almost exclusively conducted on body fluids) owing to the lack of systematic studies. For example, it is known that the amount of hydrophobic and basic drugs detected in hair strongly depends on the hair color, with dark and red hair showing higher drug incorporation than blond and white hair [19]. Nevertheless, data from doping and forensic cases involving anabolic steroids are progressively collected in the scientific literature, allowing more reliable interpretation to be made [13]. The majority of reported cases dealt with steroid administration in bodybuilding or powerlifting, resulting in quite high hair concentrations. For example, stanozolol was reported to range from 5 to 86.3 pg/mg [10,20], while metandienone from 7 to 108 pg/mg [21]. With the limitations cited above, it is common opinion that the detection of drugs at low pg/mg range in hair samples may represent single or occasional exposure to the drug, while higher levels usually indicate regular or therapeutic administration, although there is no inter-individual correlation between the frequency and dose of drug intake and hair concentration [19]. In the following paragraphs, five real cases involving clostebol are discussed and tentatively interpreted with the help of hair analysis, three of which complemented the documentation of the AAF obtained from urine testing.

In case #1, the athlete claimed he had been in unaware contact with clostebol while applying a cream to his wife. The hair results proved that both subjects had been exposed to the banned compound, with the woman's hair showing higher concentrations than the man's. The athlete provided two types of body hair, namely arm and leg hair, which usually cover a longer detection window [22] and yield higher concentration than head hair. Considering the differences in clostebol acetate concentrations, the type of hair specimens, and the similar dark color, the hypothesis of external unaware contamination could be sustained as plausible. After the athlete appealed, these evidences were partially accepted and the sentence was reduced from 24 to 15 months.

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In case #2, the athlete admitted the use of Trofodermin® claiming she was not aware that its composition included a banned substance. Clostebol acetate was detected in only one hair segment, chronologically corresponding to the declared period of the cream use. The detected concentration was relatively high, coherently with a repeated direct application of the cream onto the severe lesion she wanted to cure. The other analyzed segment was completely negative, excluding the protracted use of clostebol outside the cited period, i.e., for anabolic purposes. After the athlete appealed, these evidences were partially accepted and the sentence was reduced from 24 to 15 months.

In case #3, the athlete could not provide any explanation for the positive result, nor any other possible source of unaware clostebol intake was identified after the analysis of several pharmaceuticals and supplements habitually or occasionally used by the athlete. However, all hair samples collected from the athlete turned out negative for clostebol and clostebol acetate, unlike the cases previously described, including case #1, where exposure to clostebol had been limited and transitory. Therefore, the hypothesis of accidental exposure, possibly from contaminated food, was supportively sustained. The athlete was cleared from the doping violation charges.

In cases #4 and 5, the hair test was used for forensic purposes and ordered by a Judge. In both cases, the results documented clostebol use, with low concentrations in hair being likely related to occasional exposure. Both subjects were charged for drug diversion.

CONCLUSIONS

The detection of clostebol/clostebol acetate in consecutive hair segments can prove the regular use of this drug for anabolic purposes. On the other hand, the detection of clostebol/clostebol acetate at low concentration levels in only one hair segment, chronologically corresponding to the time of the ascertained AAF, can sustain the occasional exposure to the drug, provided that an alternative source of unaware intake is identified. Under such circumstances, the deliberate doping offense can be ruled out and the athlete's defense may obtain a charge remission. Because the IOC does not make a distinction among circumstances or means of administration of anabolic compounds, it is important that the athletes are warned against the use of clostebol-containing medications and aware of medical treatments used by their partners, especially in countries where clostebol is present in widely distributed balms and creams. While keeping the full athletes' responsability to control every potential source of banned drugs, hair analysis can nevertheless give support to those who were inadvertently contaminated, in order to produce evidence proving that their exposure had not been deliberate and in particular not aimed to improve their athletic performances. When hair analysis supports the latter claim, the athlete has the chance to obtain a diminished sanction.

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Table 1. SRM transitions and corresponding potentials for the target compounds and internal standard detection

Compound	RT (min)	Precursor Ion $[M+H]^+$	DP (V)	Target			Qualifier 1			Qualifier 2		
				Fragment	CE (V)	CXP (V)	Fragment	CE (V)	CXP (V)	Fragment	CE (V)	CXP (V)
Clostebol	4.9	323.1	87	143.0	33	14	131.0	33	5	269.3	24	9
Clostebol Acetate	6.4	365.1	81	143.0	38	10	305.2	21	5	323.2	23	12
Testosterone-d_3	4.3	292.2	47	97.2	26	5						

DP, Declustering potential; CE, Collision Energy; CXP, Cell Exit Potential.

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Table 2a. Results of calibration data and linearity tests (Adjusted Regression Coefficient R^2, Lack-of-Fit, ANOVA and Mandel's test) over the calibration interval (i.e., 1-100 pg/mg); LOD and LOQ values calculated by the Hubaux-Vos' method.

Target analyte	Model	Adjusted R^2	Lack-of-Fit (F_{crit} = 2.60)	ANOVA test (F_{tab}=3.84)	Mandel's test (F_{crit}= 2.12)	LOD (pg/mg)	LOQ (pg/mg)
Clostebol	Linear (weighted, $1/x^2$)	0.9995	0.26	0.91	1.35	0.3	0.6
Clostebol acetate	Linear (weighted, $1/x^2$)	0.9942	3.48	1.15	1.94	1.1	2.1

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Table 2b. Intraday/Interday precision (CV%) and accuracy (bias%) for the analytes tested

	Clostebol				Clostebol Acetate			
	Intraday		Interday		Intraday		Interday	
Calibration level	**Precision (CV%)**	**Accuracy (bias%)**	**Precision (CV%)**	**Accuracy (bias%)**	**Precision (CV%)**	**Accuracy (bias%)**	**Precision (CV%)**	**Accuracy (bias%)**
Low level (1 pg/mg)	20	−9	30	−8	14	−4	15	−1
Medium Level (25 pg/mg)	12	−1	9	−1	12	−4	12	−4
High Level (100 pg/mg)	6	−1	14	−1	9	−3	14	−2

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Table 3 Summary of results obtained from real cases

Case, Subject	Specimen	Total Length (cm)	Analyzed Length (cm)	Clostebol	Clostebol acetate
#1, boxer	Arm hair	3	Full length	Negative	**3 pg/mg**
#1, boxer	Leg hair	3	Full length	Negative	**5 pg/mg**
#1, boxer's wife	Head hair	12	Segment 0-2	Negative	Negative
			Segment 2-5	Negative	**13 pg/mg**
			Segment 5-12	Negative	Negative
#2, runner	Head hair	10	Segment 0-2	Negative	**21 pg/mg**
			Segment 2-10	Negative	Negative
#3, diver	Head hair	2	Segment 0-1	Negative	Negative
			Segment 1-2	Negative	Negative
#3, diver	Pubic hair	3	Full length	Negative	Negative
#4, amateur	Head hair	3	Full length	Negative	**9 pg/mg**
#5, alleged rapist	Head hair	4	Full length	Negative	**6 pg/mg**

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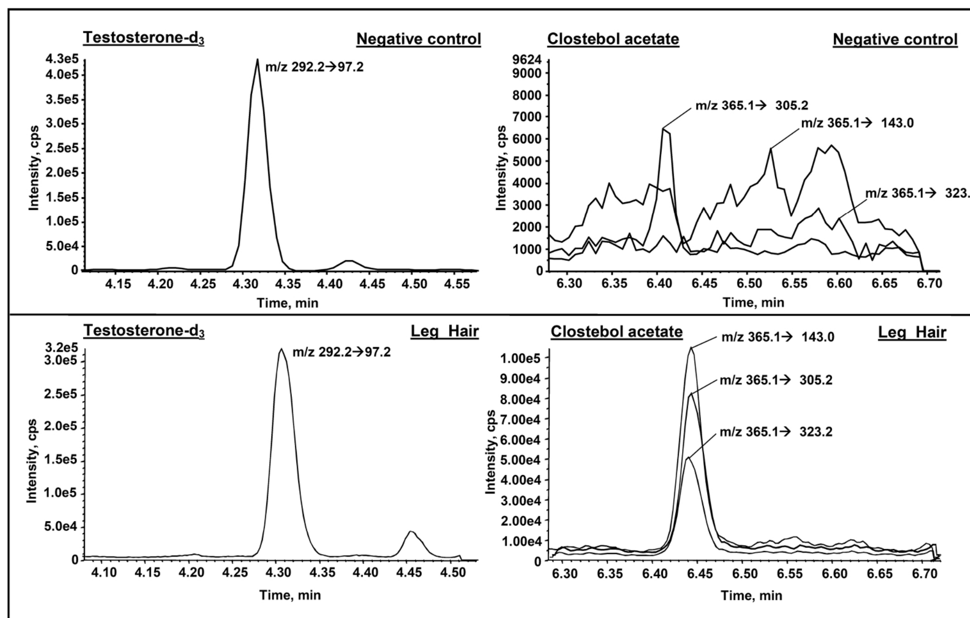

Figure 1. LC-MS/MS chromatogram of the positive clostebol acetate finding in the leg hair sample from case #1 (below) compared to a negative control (above). The chromatograms of deuterated internal standard is also shown.

185x118mm (300 x 300 DPI)