CASE REPORT

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Hair Analysis of Seven Bodybuilders for Anabolic Steroids, Ephedrine, and Clenbuterol

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ABSTRACT: Several bodybuilders, all winners of international competitions, were arrested for trafficking of a number of doping agents including anabolic steroids, ephedrine, beta-adrenergics, human chorionic gonadotropin, antidepressants, and diuretics.

In accordance with the recent French law against doping, the judge asked to test seven bodybuilders to identify doping practices. Hair and urine specimens were collected for analysis. After decontamination, a 100 mg hair strand was pulverized in a ball mill, hydrolyzed, extracted, and derivatized to be tested by GC/MS for anabolic steroids, beta-adrenergic compounds, ephedrine, and other doping agents. Urine was analyzed for anabolic steroids and metabolites, beta-adrenergic compounds, ephedrine, and human chorionic gonadotropin, in addition to a broad spectrum screening with GC/MS.

The following compounds were detected in urine: ephedrine (29 and 36 ng/mL, $n = 2$), clenbuterol (0.2 to 0.3 ng/mL, $n = 3$), norandrosterone (4.7 to 100.7 ng/mL, $n = 7$), norethiocholanolone (0.9 to 161.8 ng/mL, $n = 6$), stanozolol (1 to 25.8 ng/mL, $n = 4$), methenolone (2.5 to 29.7 ng/mL, $n = 4$), testosterone (3 to 59.6 ng/mL, $n = 7$), epitestosterone (1 to 20.4 ng/mL, $n = 7$) and ratio testosterone/epitestosterone >6 for four subjects (18.5 to 59.6).

The following drugs were detected in hair: ephedrine (0.67 and 10.70 ng/mg, $n = 2$), salbutamol (15 to 31 pg/mg, $n = 3$), clenbuterol (15 to 122 pg/mg, $n = 6$), nandrolone (1 to 7.5 pg/mg, $n = 3$), stanozolol (2 to 84 pg/mg, $n = 4$), methenolone (17 and 34 ng/ml, $n = 2$), testosterone enanthate (0.6 to 18.8 ng/mg, $n = 5$), and testosterone cypionate (3.3 to 4.8 ng/mg, $n = 2$). These results document the doping practice and demonstrate repetitive exposure to anabolic compounds and confirm the value of hair analysis as a complement to urinalysis in the control of doping practice.

KEYWORDS: forensic science, bodybuilders, doping, hair analysis, anabolics, clenbuterol, ephedrine

"Stronger, faster, further and for longer time." Man has always tried to improve his performance. This is particularly true in sports where regular doping is highlighted when testing is performed. There are many different doping substances depending on the sport and its required effort. As synthetic derivatives of testosterone, anabolic steroids promote the development of secondary male sexual characteristics and accelerate muscle growth. Athletes use steroids because they increase lean body mass, increase strength, increase aggressiveness, and lead to a shorter recovery time between workouts (1–3).

β-adrenergic compounds (agonists and antagonists) are used for their sympathomimetic properties (stimulant effects) and for their activity as anabolic agents at higher dosages, because they decrease lipogenesis and increase lipolysis and glycogenolysis. Simultaneously, they decrease protein turnover by reducing protein metabolism. Clenbuterol is used to increase the muscle mass of animals for consumption, with the potential problem of human poisoning. Human athletes use salbutamol extensively for its therapeutic properties as a bronchodilator (for asthmatics) and also to increase respiratory capacity (3,4). Ephedrine, an amphetaminic derivative, is used for its psycho stimulating properties. Human chorionic gonadotropin helps to offset the androgenic effects of steroids. Antidepressant drugs are used to reduce aggressiveness caused by androgenic derivatives. Diuretics increase urinary elimination of xenobiotics. Bodybuilders specifically use them during competition to perfect muscular definition (2).

The Medical Code of the International Olympic Committee (IOC) defines the procedures for antidoping tests and the banned and restricted classes of substances in the latest 2000 IOC list. Tests must be done on a urine sample in an accredited laboratory (6,7).

On the 23rd of March 1999, France passed a law dedicated protecting an athlete's health and fighting doping practices (Loi Buffet). As of January 11, 2001, this law allows hair as a valid specimen for doping practice evaluation (8).

In addition to conventional urinalysis, hair analysis is known to provide evidence of repeated and long-term exposure, with a longer detection window (9,10).

Recently, attempts have been made on scalp hair to demonstrate the value of this matrix as a possible means for differentiating between therapeutic use and doping abuse. Naturally occurring molecules, like testosterone and its metabolites, could also be differentiated from their synthetic counterparts, i.e., by the unique identification of the esters (10).

At the same time, several forensic cases involving doping agents were reported in the cycling. To document the pattern of drug use, the judges in charge of these cases requested toxicological analyses

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based on blood, urine, and hair tests. These tests have demonstrated the interest of doping control through hair analysis (11,12).

For practical purposes, the two tests complement each other. Urinalysis provides short-term information on an individual's drug use, whereas long-term histories are accessible through hair analysis.

The results presented in this article confirm the value of hair analysis in addition to urinalysis and highlight the extensive doping practices by bodybuilders.

Case Report

A 43-year-old bodybuilder and gymnasium leader was arrested at the Spanish border with several ampoules and tablets of anabolic steroids, ephedrine, and other drugs, that he just bought in a Spanish pharmacy. After the police investigation, the homes of several bodybuilders (customers of this gymnasium) were carefully searched. All of the bodybuilders were winners of international competitions.

Police investigations resulted in the seizure of numerous drugs such as anabolic steroids, ephedrine, human chorionic gonadotropin (HCG), antidepressants, diuretics, etc. To enforce the French law "Loi Buffet" relative to the protection of the health of the athlete (8), a judge asked the toxicologists to identify possible doping practices among seven male bodybuilders using urine and hair tests. All were Caucasians, 21 to 43 years (mean 28).

In all cases, a 40 mL urine specimen was collected by a toxicologist and stored at −20°C until analysis. Strands of hair were cut as close as possible to the skin in the vertex region and stored at ambient temperature. In all cases, length of hair that was tested was the 0 to 3 cm section from the root. A 3 cm section from the root was tested, but due to a lack of suitable hair material, no segmental analysis was performed.

Toxicological Analysis

Urinalysis

The urine specimens from the seven bodybuilders were analyzed by gas chromatography/mass spectrometry (GC/MS) using standardized methods. Sympathomimetic drugs, specifically ephedrine, were tested after alkaline extraction with ethyl acetate and derivatization using heptafluorobutyl anhydride (HFBA) (11). β-adrenergic compounds were identified after alkaline extraction and formation of methane boronate derivatives. Identification of anabolic steroids and metabolites is done after solid phase purification on C18 Isolute columns, enzymatic hydrolysis with β-glucuronidase at pH 7,6 and liquid-liquid extraction with pentane. The extract is derivatized with 50 μL of MSTFA/NH_4I/mercaptoethanol (11,13). Urinalysis of HCG was performed by a radioimmunoassay (PHARMACIA™). A screening procedure by GC-MS and liquid chromatography/diode array detector (HPLC/DAD) was also performed on urine samples.

Hair Analysis

After decontamination with methylene chloride, a 100 mg hair strand was pulverized in a ball mill, and β-adrenergic compounds were analyzed using a standardized method (14). Identification of anabolic steroids and metabolites was done after hair solubilization in NaOH 1N, solid phase purification on C18 Isolute columns, followed by a liquid-liquid extraction with pentane. The extract was derivatized with 50 μL of MSTFA/NH_4I/mercaptoethanol (15–18). Stimulants were tested after incubation in 1M NaOH, liquid-liquid extraction with ethylacetate and derivatization with HFBA (19,20). A screening procedure by GC-MS and liquid chromatography/diode array detector (HPLC/DAD) was also performed on hair samples.

Results

Results are presented in Tables 1 to 6. All the samples were tested for each class of substances described. If no results are mentioned in the tables, this means that they were under the detection limit of each compound (0.2 to 5 ng/mL for urine and 0.5 to 10 pg/mg for hair)

Discussion

According to the rules of the International Olympic Committee (IOC), the procedure to identify testosterone abuse and therefore exogenous exposure is to measure the ratio of testosterone to epitestosterone (T/E). The IOC accepts any ratio of less than 6:1 as normal (21,22).

Urinalysis of bodybuilders 3, 5, 6, and 7 (Table 1) shows a recent use of testosterone. Physiological concentrations of testosterone in human hair are available in the literature (15,23,24) and are generally in the range of 1 to 12 pg/mg. The concentrations of testosterone in the hair of Subjects 1, 3, 4, 5, and 7 are therefore largely higher than the physiological concentrations. The determination of testosterone esters (enanthate and cypionate) in the hair of both positive subjects allows a definitive and unambiguous confirmation of the administration of exogenous testosterone. Hair analysis identifies the exact nature of the parent compound and testosterone esters cannot result from an endogenous secretion (25).

Only the nandrolone metabolites norandrosterone (NA) and norethiocholanolone (NE) are identified in urine. The IOC qualifies nandrolone abuse by measuring urinary concentration of NA. The IOC accepts NA concentrations less than 2 ng/mL for male subjects and 5 ng/mL for female subjects (5), and therefore all the athletes should be considered to be abusing 19-norsteroids.

Hair specimens from Subjects 2, 4, and 5 tested positive for nandrolone, indicating chronic nandrolone abuse. In this particular case, hair analysis appears less sensitive than urinalysis to demonstrate exposure to nandrolone derivatives (Table 2).

TABLE 1—*Results for testosterone and derivatives analysis.*

	Sample	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Testosterone	Urine	3	5	39	18.6	50	57.8	59.6
Epitestosterone	Urine	1	2	2	20.4	1.2	2.1	1
Ratio T/E	Urine	3	2.5	18.5	<1	41.6	27.52	59.6
Testosterone	Hair	26.6	10.6	17.4	18.6	72.4	4.2	230
Testosterone enanthate	Hair	18.8	–	0.6	5.4	2.7	–	4.0
Testosterone cypionate	Hair	3.3	–	–	–	–	–	4.8

NOTE: Units are in pg/mg for hair analysis and in ng/mL for urinalysis.

TABLE 2—*Results for 19-norsteroids derivatives analysis.*

	Sample	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Norandrosterone	Urine	36	4.7	127	5.4	133	87.8	100.7
Norethiocholanolone	Urine	58	0.9	37	–	260	105.8	161.8
Nandrolone	Hair	–	1	–	3.5	7.5	–	–

NOTE: Units are in pg/mg for hair analysis and in ng/mL for urinalysis.

TABLE 3—*Results for other anabolic steroids and DHEA analysis.*

	Sample	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
DHEA	Urine	5	5.1	11.8	–	2.6	3.9	1.2
DHEA	Hair	10.4	10.1	33	20.4	22.5	7.1	1.6
Stanozolol	Urine	–	1	–	3.5	11.1	–	25.8
Stanozolol	Hair	3.3	1.8	–	–	23.1	–	84
Methenolone	Urine	–	–	11.6	–	4.7	29.7	2.5
Methenolone	Hair	–	–	34	–	17	–	–

NOTE: Units are in pg/mg for hair analysis and in ng/mL for urinalysis.

TABLE 4—*Results for ß-adrénergics compounds analysis.*

	Sample	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Clenbuterol	Urine	0.2	–	0.2	–	0.3	–	–
Clenbuterol	Hair	21	79	80	15	94	–	122
Salbutamol	Urine	–	–	–	–	–	–	–
Salbutamol	Hair	15	–	–	–	19	–	31

Note: Units are in pg/mg for hair analysis and in ng/mL for urinalysis.

TABLE 5—*Results for psychostimulants analysis.*

	Sample	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Ephedrine	Urine	29	–	–	–	–	36	–
Ephedrine	Hair	10 700	–	–	–	–	670	–

NOTE: Units are in pg/mg for hair analysis and in ng/mL for urinalysis.

TABLE 6—*Other components identification results.*

	Sample	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
HCG	Urine	<2	<2	<2	5	<2	<2	<2
Ketoprofene	Hair	–	–	–	–	–	+	–
Diclofenac	Hair	+	–	–	–	–	–	–

NOTE: Unit are in IU/mL for the HCG.

Dehydroepiandrosterone (DHEA) was added to the IOC list because DHEA is presented as a precursor of testosterone and estrogen (23). Physiological concentrations of DHEA in human hair are available in the literature and are generally in the range of 1 to 10 pg/mg (26). Hair DHEA concentrations from five subjects were higher than this range (Table 3), indicative of a chronic use. DHEA, classified as a dietary supplement, is available over-the-counter or through the Internet.

Stanozolol (Winstrol ™) was identified in the hair of Subjects 1, 2, 5, and 7 and in the urine of Subjects 2, 4, 5, and 7. Methenolone (Primobolan ™) was identified in the hair of Subjects 3 and 5, and in the urine of Subjects 3, 5, 6, and 7 (Table 3). Again, it seems that, in rate of positivity, urine is more appropriate than hair. This is probably due to the analytical sensitivity and the low incorporation of the drugs in hair. A chronic abuse of a β-adrenergic compound cannot be demonstrated with urinalysis. These compounds have a relatively short half-life. Both abstaining from the drug for a few days and trying to evade the test by diluting urine can lead to a negative urinalysis (9,20). Hair specimens from six subjects were positive for clenbuterol confirming the chronic use, whereas only three subjects were positive in urine, with very low concentrations (Table 4).

Human chorionic gonadotropin was not detected in urine with radioimmunoassay (detection limit: 2 U/l), except for one subject (Table 6), perhaps because this hormone has a short half-life. Due

to a large steric volume, HCG is probably not incorporated in growing cells of the hair matrix.

The Courts frequently request documentation of drug exposure through hair analysis in forensic cases. The value of hair analysis is largely documented in this field. Only two publications about doping cases in bodybuilders are available in the literature. Thieme et al. identified testosterone esters, nandrolone, methenolone, and metandienone (28) in the hair of a bodybuilder who died of cardiac infarction at an age of 32. In a forensic case involving the French customs, Kintz et al. tested two bodybuilders arrested with various ampoules and tablets of anabolic steroids. In both subjects, their hair tested positive for stanozolol, nandrolone, and testosterone, clearly demonstrating chronic exposure (17). Finally, Gaillard et al. identified amphetamine, corticosteroids, and anabolic steroids in the hair of cyclists during the 1998 cycling Tour de France (11,27).

Our results complete the list of doping compounds identified in the hair of athletes and demonstrate the value of a hair in doping control. However, with a 200 mg specimen, it is not possible to screen for the approximately 200 compounds listed by the IOC in hair like it is in urine. Several criteria can affect the detection of doping agents in hair such as hair pigmentation, ethnic origin, and cosmetic treatments (29).

It must be mentioned that negative hair results do not mean that no doping agents have been taken. It has always been accepted in the forensic community that a negative hair result cannot exclude the administration of a drug and should not overrule a positive urine result.

Conclusion

Results presented in this case report illustrate the wide variety of doping agents used by bodybuilders during training. Long-term histories of an individual's drug use are accessible through hair analysis whereas urinalysis provides only short-term information. In fact, hair tests complement tests in the mandatory sample (urine) for doping control of Olympic level athletes during both training and competition periods. In this study, hair analysis completes urinary results for demonstrating chronic use of prohibited substances in accordance with the French law (8). If according to validated standard operating procedures and if accepted by the International Olympic Committee, hair analysis may find many applications in doping control (30).

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