Nandrolone or 19-nortestosterone, or 17β-hydroxy-19-nor-4-androsten-3-one, has been one of the most abused anabolic steroids and doping practices are increasing, as revealed by numerous positive cases during the last 5 years in various sports. The drug accelerates muscle growth by an anabolic effect. Athletes use nandrolone because it has been claimed that it increases lean body mass, increases strength, increases aggressiveness and leads to a shorter recovery time between workouts.

The standard of testing for anabolic steroids for doping control is gas chromatography coupled to mass spectrometry conducted on a urine sample, and performed in accredited laboratories. The International Olympic Committee (IOC)-accredited laboratory reporting cut-off value is 2 and 5 ng/ml of norandrosterone for male and female, respectively.

There has been a controversy in the literature about the possible endogenous secretion in human of nandrolone metabolites, norandrosterone (NA) and noretiocholanolone (NE) (1-3). NA seems to be excreted in trace amounts (always < 2 ng/ml) in human urine, probably as a result of aromatase action in the formation of estrogens (1). Moreover, other 19-norsteroids, such as norandrostenedione or norandrostenediol, classified as dietary supplements, are available over-the-counter or through the Internet and have the same metabolites as nandrolone (4).

Long-term effects (severe cardio-vascular side-effects, liver diseases, etc.) and fatalities have been reported in young steroid abusers. Liver diseases such as peliosis hepatitis, cholestasis or hepatic tumors and neurologic disorders have been reported after steroids abuse (5). Moreover, as anabolic androgens have direct effects on cardiac growth, on myocyte metabolism and on platelet function, cardiovascular diseases such as myocardial infarction, sudden arrhythmic death and stroke have been described in young steroids abusers (6).

The current rules governing doping in sport have as their core that a doping violation is deemed to occur on finding in urine a prohibited substance, a metabolite of a prohibited substance or a compound chemically or pharmacologically related to a prohibited substance. An athlete is responsible for any substance that enters his or her body.

During the past years, our laboratory was involved in several cases, where an athlete declared positive, based on urinary NA findings > 2ng/ml (analysis performed in an accredited IOC
laboratory) denied the result an asked us to document the findings. Although it was not a question to overrule the positive urine result, we investigated the different possibilities to address a scientific response to the case.

Our approach was to use all the evidence available to explain how the urine could be positive, including both urine and hair analyses, food analysis and dietary supplements.

Hair specimens have been used for 20 years in toxicology to document chronic drug exposure in various forensic, occupational and clinical situations. Urinalysis provides short-term information of an individual's drug use, whereas long-term histories are accessible through hair analysis. For example, doping during training and abstinence during the competition can therefore be detected by hair analysis (7-9). However, in our laboratory, hair analysis was always achieved taking into consideration the Consensus of the Society of Hair Testing on hair testing for doping agents (10).

This paper describes our investigations in case of positive urine for NA, using various pieces of evidence as it is achieved in forensic sciences.

Material and methods

Urine analysis

Urine specimens were analyzed using our published procedure (4) using gas chromatography coupled to mass spectrometry.

Hair analysis

Hair was tested for 19-Norsteroids after modifications of our published procedure (11), mainly by developing a tandem mass spectrometry detection.

Briefly, the hair was decontaminated twice using 5 ml of methylene chloride, for 2 min at room temperature, and then pulverised in a ball mill. One hundred milligrams of decontaminated hair were incubated in 1 ml 1N NaOH, 15 min at 95°C, in presence of 1 ng of nandrolone-d3 used as internal standard. After cooling, the homogenate was neutralized with 1 ml 1M HCl, and 2 ml of 0.2 M phosphate buffer (pH 7.0) were added.

The Isolute C18 columns were conditioned with 3 ml of methanol, followed by 2 ml of deionized water. After sample addition, the columns were washed twice with 1 ml of deionized water.

After column drying, analyte elution occurred with the addition of 3 aliquots of 0.5 ml of methanol. The eluant was evaporated to dryness under nitrogen flow, and the residue reconstituted in 1 ml of 0.2 M phosphate buffer (pH 7.0). A further purification step was achieved by addition of 100 mg of
Na2CO3/NaHCO3 (1:10, w/w) and 2 ml of pentane. After agitation and centrifugation, the organic phase was removed and evaporated to dryness. The residue was derivatized by adding 50 µl MSTFA/NH4I/2-mercaptoethanol (1000 : 2 : 5 ; v/v/v), then incubated for 20 min at 60 °C.

A 2-µl aliquot of the derivatized extract was injected into the column of a Hewlett Packard gas chromatograph (5890 Series). The flow of carrier gas through the column (HP5-MS capillary column, 5 % phenyl-95 % methylsiloxane, 30 m x 0.25 mm i.d. x 0.25 mm film thickness) was 1.0 ml/min.

The injector temperature was 270 °C and splitless injection was employed with a split valve off-time of 1.0 min. The column oven temperature was programmed to rise from an initial temperature of 150 °C, maintained for 1 min, to 295 °C at 5 °C/min.

The detector was a Finnigan TSQ 700 operated in the electron impact mode and in selected reaction monitoring. The parent ions, m/z 416, 420, 418, and 421 for norandrostenedione, norandrostenediol, nandrolone, and the IS, respectively, were selected in the first quadrupole. The corresponding daughter ions, m/z 220 and 234, 182 and 240, 182 and 194, and 136 for norandrostenedione, norandrostenediol, nandrolone, and the IS, respectively, were selected in the third quadrupole after collision with argon at a cell pressure at 0.6 mTorr. The collision offset voltage was - 8 V (norandrostenedione, norandrostenediol), or - 13 V (nandrolone, IS). The electron multiplier was operated at 1900 V.

Limits of detection were 5, 1 and 0.5 pg/mg for norandrostenedione, norandrostenediol, and nandrolone, respectively.

Current knowledge

Although norandrostenediol and norandrostenedione (available over-the-counter or through the Internet) are banned by the IOC, there is a great need in forensic science and for survey of the athletes, to discriminate nandrolone from other 19-norsteroids. This is obviously not possible in urine, as the metabolites are common. Hair can identify the exact nature of the parent compound (e.g., nandrolone, norandrostenediol or norandrostenedione, in case of positive urine for NA), as it has been accepted by the scientific community that the parent compound is the major analyte that is incorporated in hair. Thus, hair analysis would discriminate nandrolone abuse from over-the-counter preparations containing 19-norsteroids. Recently, our laboratory was requested by an attorney to evaluate potential doping practices from an athlete. The 30-year old subject tested positive for norandrosterone in urine at 230 ng/mL. The analysis was done in an accredited laboratory, but the athlete denied the result. The analysis of a strand of hair obtained from the athlete revealed the presence of 19-norandrostenedione at the concentration of 7 pg/mg (11), making an unique distinction with nandrolone doping.
When using hair in a suspected doping case, particularly when urine of the athlete was positive and hair negative (several cases were reported during the past years), the question of importance is to know whether the analytical procedure was sensitive enough to identify traces of drugs. It has been always accepted in the forensic community that a negative hair result cannot exclude the administration of the detected drug or one of its precursors (such as norandrostenediol or norandrostenedione for the metabolites of nandrolone) and should not overrule a positive urine result. Nevertheless, the negative hair findings lend enough ambiguity to the positive urine result, coupled with the sporting consequences for the athlete, that substantial justice refereeing occurs.

This laboratory was not able to identify nandrolone in the hair of a 37-year old man, receiving a single intramuscular injection of 50 mg nandrolone undecanoate, although his urine remained positive for norandrosterone and noretiocholanolone, the nandrolone metabolites, for at least 8 months. Hair was tested 2 and 6 months after administration (12). The same observations were recently made by Segura, et al. (13) who did not detect nandrolone after a single dose administration. Therefore, until laboratories will have sensitive enough methodologies to detect a single use of steroids, care should be taken to compare urine and hair findings. For anabolic steroids to have an appreciable performance enhancing effect, they must be chronically administered. Repeated amount of drug used per hair growth length would favor identification by hair analysis.

In case of doping control, drugs are screened in urine specimens according to validated standard operating procedures in accredited laboratories. As forensic laboratories can be involved in testimony dealing with doping agents, the idea of using hair for doping control has emerged as hair analysis has been accepted in court in other cases. Courts can request additional informations on the pattern of use of doping substances, such as during the 1998 cycling Tour de France where blood, urine, and hair were simultaneously collected. Hair can both confirm repetitive abuse and identify the exact nature of the parent compound.

However, some issues have to be discussed before considering hair as a valid specimen by the I.O.C. and the International Sport Federations (10). The relationship between urine and hair results is not yet established and negative hair result does not mean « no doping ».

After intra-muscular administration of nandrolone ester, NA is detectable for over 8 months in urine, using a positive cut-off at 2 ng/ml (4). After the 2nd month, the slope of the elimination rate of NA is very weak. Therefore, the NA concentration in urine will not vary too much over a period of several weeks and sampling urine several days after a positive control will aid to discriminate the chemical structure of nandrolone. Authentic nandrolone or orally administered norandrostenediol or norandrostenedione will be rapidly (4-8 days) cleared from the body. On the opposite, an ester preparation injection will be detectable in urine for months.
Concerns have been raised that NA may be found in urine after ingestion of contaminated food products. Ingestion of meat containing nandrolone injection site (14) or noncastrated male pigs (15) can result in NA urinary concentrations > 2 ng/ml.

Contamination of dietary supplements (vitamins, creatine, salts ...) by 19-norsteroids or precursors, as a result of impurities from the synthetic process, has also been described (16). However, according IOC, the inadvertent ingestion of contaminated products, cannot be used as an evidence or even as an excuse, because under the rules of sports the athlete has strict liability for any substance entering his body.

**Forensic investigations**

The first step is to take into consideration the measured NA concentration in the doping control specimen. Concentrations higher than 50 ng/ml are highly indicative of doping practice. Most cases we were involved were in the range 3 to 15 ng/ml.

The first step, when an athlete denies a positive result, is to collect a fresh urine specimen and analyze it for NA. If NA > 2 ng/ml (when urine is collected some weeks after the doping control), the result is highly indicative of doping. If NA < 2 ng/ml, then hair testing is requested.

Hair analysis will help in demonstrating chronic exposure or not. On the opposite to amphetamine or cocaine, which can be used as a single exposure to get stimulation, anabolic steroids must be taken chronically to have an effect. A positive hair test will document doping and allow discrimination between norandrostenediol, norandrostenedione or nandrolone. A negative hair test can be interpreted as follows : 1. no chronic anabolic exposure; 2. single anabolic exposure (as the hair test is not sensitive enough to detect a single exposure, even by using tandem mass spectrometry) but this is a non-sense as nobody will use anabolics just for 1 time; or 3. contamination by food or dietary supplements. From our experience, the contamination by dietary supplements is the more frequent. Analyses of all the ingested supplements will aid in getting suitable informations.

**Conclusion**

Although hair is not yet a valid specimen for the International Olympic Committee (IOC), it is accepted in most courts of justice. Some conflicting results have been observed, all involving athletes that tested positive in urine at accredited IOC laboratories and negative in hair in forensic certified laboratories.
Much experience has been acquired in the detection of opiates and cocaine in hair. In contrast, there is a serious lack of suitable references to interpret the analytical findings for doping agents. In hair, doping agent concentrations, such as anabolic steroids, are in the range of picograms per milligram, whereas drugs of abuse are generally found in the range of several nanograms per milligram.

More research is required before all of the scientific questions associated with hair drug testing will be satisfied. There is still a lack of consensus among the active investigators on how to interpret the analysis of drugs in hair. Amongst the unanswered questions, five are of critical importance: 1. What is the minimal amount of drug detectable in hair after administration? 2. What is the relationship between the amount of the drug used and the concentration of the drug or its metabolites in hair? 3. What is the influence of hair colour? 4. What is the influence of genetic differences in hair testing? 5. What is the influence of cosmetic treatments? Several answers were recently addressed (12) on these specific topics.

References


