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Short communication

Doping control for nandrolone using hair analysis

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Abstract

A sensitive, specific and reproducible method for the quantitative determination of nandrolone in human hair has been developed. The sample preparation involved a decontamination step of the hair with methylene chloride. The hair sample (about 100 mg) was solubilized in 1 ml NaOH IN, 15 min at 95°C, in presence of 10 ng nandrolone-d_3 used as an internal standard. The homogenate was neutralized and extracted using consecutively a solid phase (Isolute C18) and a liquid–liquid (pentane) extraction. 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 4-μl aliquot of the derivatized extract was injected into the column (HP5-MS capillary column, 5% phenyl–95% methylsiloxane, 30 m × 0.25 mm i.d. × 0.25 mm film thickness) of a Hewlett Packard (Palo Alto, CA) gas chromatograph (6890 Series) via a Hewlett Packard (7673) autosampler. The assay was capable of detecting 0.5 pg of nandrolone per mg of hair when approximately 100 mg of hair were processed. Linearity was observed for nandrolone concentrations ranging from 1 to 50 pg/mg with a correlation coefficient of 0.997. Intra-day and between-day precisions at 10 pg/mg were 11.2 and 15.1%, respectively, with an extraction recovery of 81.7%. The analysis of three strands of hair, obtained from three bodybuilders, revealed the presence of nandrolone at the concentration of 1, 3.5 and 7.5 pg/mg.

Keywords: Nandrolone; Doping; Hair; Abuse; Steroid

1. Introduction

Androgen use was widespread among athletes at the time of the 1964 Olympic Games [1], and, despite a ban by the International Olympic Committee (IOC) in 1974, the use of anabolic steroids increased during the last decade.

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 3 yr in various sports. The drug accelerates muscle growth by an anabolic effect. Athletes use nandrolone because

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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 an urine sample, and performed in accredited laboratories. There has been a controversy in the literature about the possible endogenous secretion in human of nandrolone metabolites, norandrosterone (NA) and noretiocholanolone (NE) [2,3]. Abuse of anabolic steroids can be difficult to detect in urine because they are frequently taken for periods ranging from 4 to 18 weeks, alterning with drug-free periods of 1 month to 1 yr [4]. 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 [5].

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 hepatis, cholestasis or hepatic tumors and neurologic disorders have been reported after steroids abuse [6]. 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 [7,8].

Hair specimens have been used for 20 yr in toxicology to document chronic drug exposure in various forensic, occupationnal and clinical situations [9]. 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 [10,11].

This paper describes a very sensitive and specific analytical method for the detection and quantification of nandrolone in human hair by gas chromatography–mass spectrometry.

2. Materials and methods

2.1. Specimen

Hair strands were obtained from three body builders who were arrested by the French customs, accused of trafficking. In their luggage, the officers discovered a large amount of anabolics in ampoules. Full-length hair samples (3–5-cm long) were taken at the surface of the skin from the vertex and stored in plastic tubes at room temperature. Controlled hair specimens were obtained from laboratory personal.

2.2. Chemicals and reagents

Dichloromethane, pentane and methanol were HPLC grade (Merck, Darmstadt, Germany). All other chemicals were of analytical grade and provided by Merck. Isolute C18 columns were purchased from Touzart et Matignon (Courtaboeuf, France). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), 2-mercaptoethanol and ammonium iodide (NH_4I) were purchased from Fluka (Saint-Quentin Fallavier, France). Nandrolone and nandrolone-d_3 were purchased from Cambridge Laboratories (Andover, MD).

2.3. Nandrolone extraction

The hair was decontaminated twice using 5 ml of methylene chloride, for 2 min at room temperature, and then pulverized in a ball mill.

One hundred milligrams of decontaminated hair were incubated in 1 ml of 1 N NaOH, 15 min at 95°C, in presence of 1 ng of nandrolone-d_3 used as internal standard. After cooling, the homogenate was neutralized with 1 ml of 1 M 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 occured with the addition of 3 aliquots of 0.5 ml of methanol. The eluant was evaporated to dryness under nitrogen flow, and the residue reconstitued in 1 ml of 0.2

M phosphate buffer (pH 7.0). A further purification step was achieved by addition of 100 mg of Na_2CO_3/$NaHCO_3$ (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/NH_4I/2-mercaptoethanol (1000:2:5 v/v/v), then incubated for 20 min at 60°C.

2.4. GC-MS procedure

A 4-μl aliquot of the derivatized extract was injected into the column of a Hewlett Packard (Palo Alto, CA) gas chromatograph (6890 Series) via a Hewlett Packard (7673) autosampler. The flow of carrier gas (helium, purity grade N 55) through the column (HP5-MS capillary column, 5% phenyl–95% methylsiloxane, 30 m × 0.25 mm i.d. × 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, using the pulsed mode. The column oven temperature was programmed to rise from an initial temperature of 150°C, maintained for 1 min, to 295°C at 30°C/min and maintained at 295°C for the final 8 min.

The detector was a Hewlett Packard 5973 operated in the electron impact mode. The electron multipler voltage was set at 600 V above the EI-tune voltage.

2.5. Method validation

A standard calibration curve was obtained by adding 0.1 ng (1 pg/mg), 0.5 ng (5 pg/mg), 1 ng (10 pg/mg), 2 ng (20 pg/mg), and 5 ng (50 pg/mg) of nandrolone to 100 mg of powdered control hair (negative for nandrolone).

Within-run and betwenn-run precisions for nandrolone were determined using negative control hair spiked with nandrolone at the final concentration of 10 pg/mg ($n = 6$). Relative extraction recovery was determined by comparing the representative peak area of nandrolone extracted from negative control hair spiked at the final concentration of 10 pg/mg with the peak area of a methanolic standard at the same concentration. The detection limit (LOD) was evaluated with decreasing concentrations of nandrolone until a response equivalent to 3 times the background noise was observed.

3. Results and discussion

3.1. Validation results

Under the chromatographic conditions used, there was no interference with the analytes by any extractable endogenous materials present in hair. The mass spectrum of nandrolone is shown in Fig. 1. Selected ions and retention times of nandrolone and the deuterated internal standard are reported in Table 1. Analytes were identified and quantified on the basis of their retention times and the abundance of three confirming ions. Responses for nandrolone were linear in the range 1–50 pg/mg, with a correlation coefficient of 0.997.

The within-run precision was 11.2%, as determined by analyzing 6 replicates of 100 mg of hair obtained from the same subject and spiked with a nandrolone final concentration at 10 pg/mg. The between-run precision was 15.1%, as determined by analyzing during 6 consecutive days, replicates of hair from the same subject spiked at 10 pg/mg. The extraction recovery ($n = 3$) was determined to be 81.7%. The limit of detection of nandrolone was 0.5 pg/mg.

3.2. Application

The analysis of three strands of hair, obtained from three body buiders revealed the presence of nandrolone at the concentrations of 1, 3.5 and 7.5 pg/mg. Fig. 2 is the chromatogram obtained in single ion monitoring mode of one of the athlete. The nandrolone concentration was 3.5 pg/mg.

Extensive chromatographic procedures (two purification steps by solid phase and liquid–liquid extractions, combined with injection of 4 μl through the column in pulsed mode) were analytical prerequisites for successful identification of nandrolone in hair due to the low target concentrations.

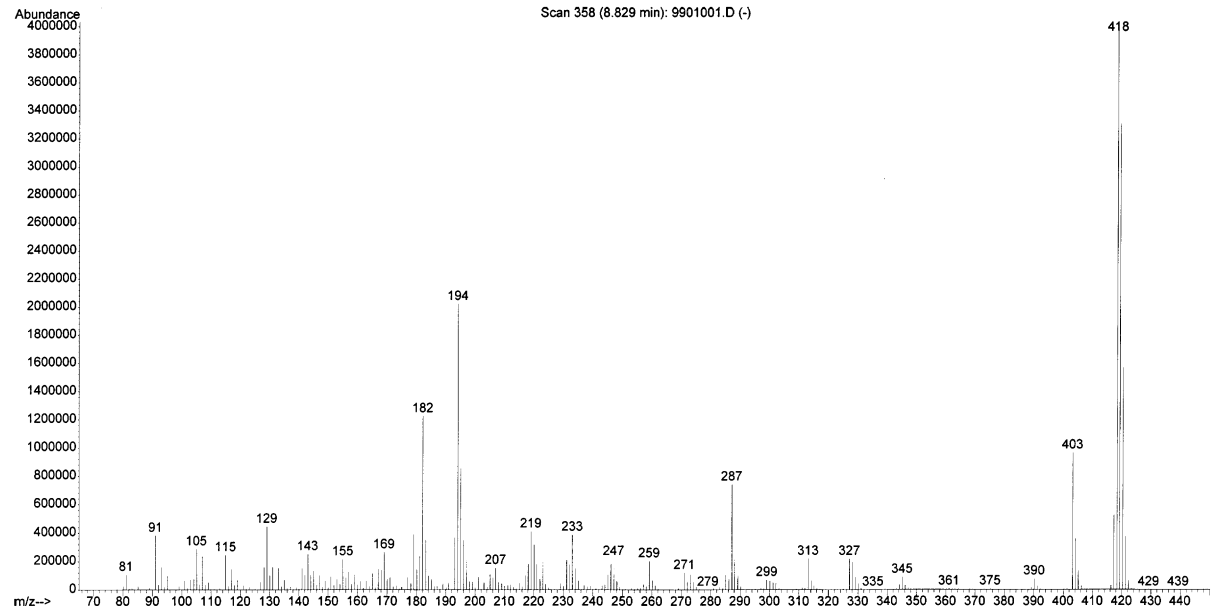

Fig. 1. Mass spectra of TMS-derivative of nandrolone.

3.3. Discussion

The international literature is very poor in papers dealing with the identification of nandrolone in hair. One paper, published in 1995, has been focused on guinea pigs [12] with unrealistic nandrolone dosages, 10 or 20 mg/kg. In 1999, Höld et al. [13] demonstrated that it is possible to detect nandrolone in rat hair after systemic administration. However, the growing interest of scientists to the detection of anabolic drugs in human was observed in the late 1998 [10,14,15].

In their early work, Cirimele et al. [16], published results from two body builders preparing the next world championship, with positive hair for nandrolone at 196 and 260 pg/mg. Deng et al. [14], among other anabolics, identified nandrolone in the hair of a steroid abuser at 20 pg/mg. Gaillard et al. [17] detected nandrolone in the hair of a cyclist at 5.1 pg/mg.

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 3 yr), 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 lends enough ambigity to the positive urine result, coupled with the sporting consequences for the athlete, that substancial justice refereeing occurs.

This laboratory was not able to identify nandrolone in the hair of a 37-yr-old man, receiving a single intramuscular injection of 50 mg nandrolone undecanoate, although his urine remained positive for NA and NE, the nandrolone metabolites, for at least 8 months. Hair was tested 2 and

Table 1
Selected ion (m/z) and retention times for nandrolone and its deuterated analog[a]

Analyte	Retention time (min)	Ions (m/z)
Nandrolone	7.17	287-403-*418*
Nandrolone-d_3	7.16	*421*

[a] The italicised ions were used for quantification.

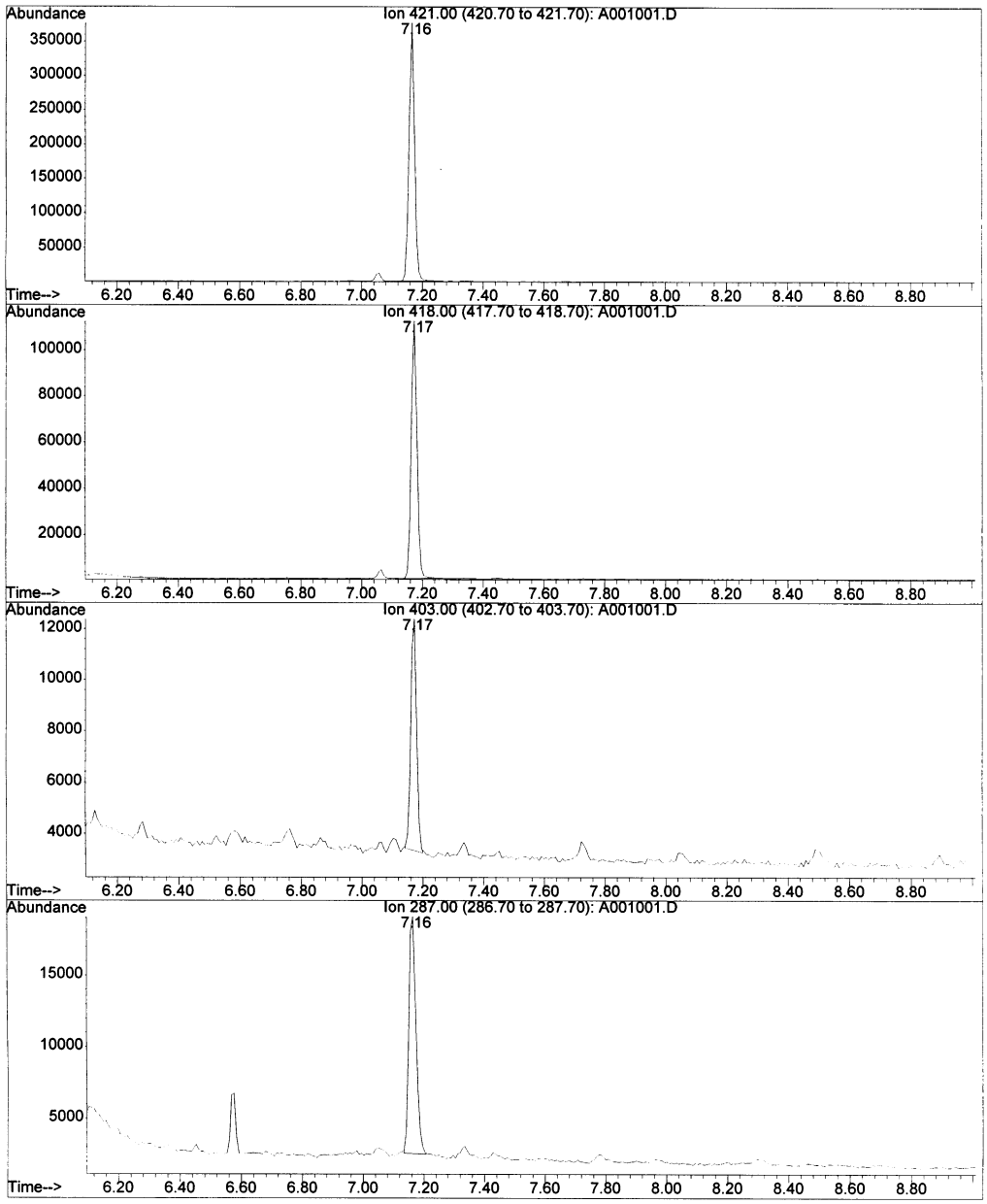

Fig. 2. SIM chromatogram obtained after extraction by the established procedure of a 98-mg hair specimen of a body builder. Nandrolone was identified and quantified at the concentration of 3.5 pg/mg.

6 months after administration [11]. The same observations were recently made by Segura et al. [18] 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, in contrast to the immediate stimulant

properties of cocaine or amphetamine. 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 information 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 (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, although in urine this distinction is not possible [19].

However, some issues have to be discussed before considering hair as a valid specimen by the IOC and the International Sport Federations [20]. The relationship between urine and hair results is not yet established and negative hair result does not mean *no doping*.

4. Conclusion

The sensitive, specific and reproducible method developed seems to be suitable for the detection and quantification of nandrolone in human hair.

Hair analysis may be a useful adjunct to conventional drug testing in sports. Methods for evading urine analysis do not affect the drug concentrations in hair. Specimens can be more easily obtained with less embarrassment, and hair can provide a more accurate history of drug use. This technology may find useful applications in doping control, if accepted by the International Olympic Commitee.

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