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Short communication

Doping control for metandienone using hair analyzed by gas chromatography–tandem mass spectrometry

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Abstract

A sensitive, specific and reproducible method for the quantitative determination of the anabolic metandienone in human hair has been developed. The preparation involved a decontamination step with methylene chloride. The hair sample (about 50 mg) was solubilised in 1 ml 1 M NaOH, 10 min at 95 °C, in presence of 2 ng of nandrolone-d_3 used as internal standard. The homogenate was neutralized and extracted using consecutively a solid-phase extraction (Isolute C_{18} eluted with methanol) and a liquid–liquid extraction with pentane. The residue was derivatized by adding 5 μl MSTFA/NH_4I/2-mercaptoethanol (250 μl; 5 mg; 15 μl) and 45 μl MSTFA, then incubated for 20 min at 60 °C. A 1 μl aliquot of derivatized extract was injected into the column (HP5-MS capillary column, 5% phenyl–95% methylsiloxane, 30 m × 0.32 mm i.d., 0.25 μm film thickness) of a Hewlett Packard (Palo Alto, CA, USA) gas chromatograph (6890 Series). Metandienone was identified using three transitions (its daughter ions at *m/z* 339 and 206 for the parent 444 and 191 for 206) using a Waters Quattro Micro MS–MS system. The transition *m/z* 444 to 206 has been used as quantification transition and the others as identification transitions. The assay was capable of detecting 2 pg/mg of metandienone when approximately 50 mg of hair material was processed. Linearity was observed for metandienone concentrations ranging from 2 to 500 pg/mg with a correlation coefficient of 0.9997. Intra-day and between-day precisions at 50 pg/mg were 13.4–16.5% and 22.0%, respectively, with an extraction recovery of 48%. The analysis of hair, cut into four segments, obtained from an athlete, revealed the presence of metandienone at the concentrations of 78, 7, 10 and 108 pg/mg in each segment of hair (0–1, 1–2, 2–3 and 3 cm to the tip).

Keywords: Metandienone; Doping; Hair; MS–MS

1. Introduction

Metandienone, or 17β-hydroxy-17α-methylandrosta-1,4-dien-3-one is an exogenous anabolic androgenic steroid. Anabolic steroids are synthetic compounds structurally related to testosterone, the male sex hormone.

The drug is available in tablets under the trade name Dianabol® since 1956. It was reported as being the first anabolic steroid used by athletes [1]. Dianabol® is one of the most commonly abused oral steroid. This drug accelerates muscle growth by an anabolic effect. Athletes can abuse metandienone because it has been claimed that it increases lean body mass, strength, aggressiveness and leads to a shorter recovery time between workouts.

Use of anabolic steroids was officially banned in the mid-1970s by sports authorities. The first control of anabolic steroids was achieved in Montreal in 1976 during the Olympic games.

The detection of anabolic steroids misuse in sports is based on the analysis of urine samples. But some athletes take long-term treatment of anabolic steroids during the winter months and stop before the competition [2] or for periods ranging from 4 to 18 weeks, altering with drug-free periods of 1 month to 1 year [3]. This is the reason why they are often found urine-free as anabolic steroids are detectable in urine only 2–4 days after exposure, except for the ester forms.

Hair specimens have been used for 20 years in toxicology to document chronic exposure in various forensic, occupational and clinical situations [3].

Hair analysis allows an increase in the detection window (weeks to months) depending on the hair length. It allows to make a distinction between a single or a repetitive use and documents an estimation of consumed quantities [4–7]. It is for that

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reason that doping during training and abstinence during competition can be detected by hair analysis.

There are very few papers in the international literature dealing with the identification of metandienone. The sample was prepared according to the procedure described by Kintz et al. [8] which has already been published and which uses the same method as the one recommended by the antidoping laboratories WADA.

The aim of this work was to develop a sensitive, specific and reproducible method to detect and quantify metandienone in human hair of an athlete.

2. Materials and methods

2.1. Specimens

Hair strands were obtained from an athlete who requested us for an expertise after he has been reported positive in urine. Full-length hair samples (5 cm long) were taken at the surface of the skin from the vertex and stored in a envelope at room temperature. Hair was bleached by the athlete 15 days before sampling.

Controlled hair-free specimens were obtained from laboratory personnel.

2.2. Chemicals and reagents

Dichloromethane, methanol and ammonium iodide (NH_4I) were from Carlo ERBA (Val de Reuil, France). All other chemicals were of analytical grade and provided by Merck.

Isolute C_{18} columns were purchased from Interchim (Montlucon, France).

N-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), 2-mercaptoethanol and pentane were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France).

Metandienone and nandrolone-d_3 were purchased from LGC Promochem (Molsheim, France).

2.3. Metandienone extraction

The sample was prepared according to the procedure described by Kintz et al. [8].

The hair was decontaminated twice using 15 ml of dichloromethane, for 2 min at room temperature. It was segmented in four segments, of 1 cm for the three first cm and 2 cm for the last segment.

A 50 mg of decontaminated hair was incubated in 1 ml 1 M NaOH, 10 min at 95 °C, in the presence of 2 ng of nandrolone-d_3 (final concentration 40 pg/mg) used as internal standard. After cooling, the homogenate was neutralized with 1 ml 1 M HCl and 2 ml of 1 M phosphate buffer (pH 7.0) were added.

The Isolute C_{18} columns were conditioned with 3 ml of methanol, followed by 3 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 three 0.5 ml aliquots of methanol. The eluent was evaporated to dryness, and the residue reconstituted in 1 ml of 1 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 5 μl MSTFA–NH_4I–2-mercaptoethanol (250 μl, 5 mg, 15 μl, respectively) and 45 μl MSTFA, then incubated for 20 min at 60 °C.

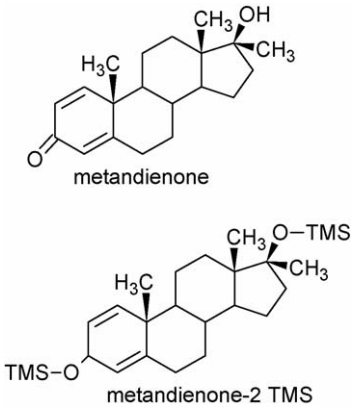

Fig. 1. Structure of metandienone and metandienone-2 TMS.

2.4. GC–MS–MS procedure

A 1 μl aliquot of derivatized extract was injected into the column of a Hewlett-Packard (Palo Alto, CA, USA) gas chromatograph (6890 Series). The flow of carrier gas (helium, purity grade 99.9996%) through the column (HP5-MS capillary column, 5% phenyl–95% methylsiloxane, 30 m × 0.32 mm i.d, 0.25 μm film thickness) was 1.5 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 60 to 295 °C at 30 °C/min and maintained at 295 °C for the final 10 min.

The detector was a Waters Quattro Micro operated in the electron impact and in selected reaction monitoring modes. The parent ions, *m*/*z* 444 and 206 for metandienone and 421 for the I.S. were selected in the first quadrupole. The parent ion 444 is the molecular ion of metandienone on which is added two molecules of TMS (Fig. 1). Parent ion 206 is an ion found in all spectrum of steroids after passage in GC–MS. The parent ions have been chosen according to their abundance on the spectrum and their specificity.

The corresponding daughter ions, *m*/*z* 339, 206 and 191 for metandienone and 194 for I.S., respectively, were selected in the third quadrupole after collision with argon at a cell pressure of 1.00×10^{-4} Pa. The collision offset voltage was 15 eV for the transitions *m*/*z* 421.4–194.2 and 444.2–206.1, and 10 eV for other transitions. The electron multiplier was set at 650 V.

2.5. Method validation

A standard calibration curve was obtained by adding 0.1 ng (2 pg/mg), 0.25 ng (5 pg/mg), 0.75 ng (15 pg/mg), 2.5 ng

(50 pg/mg) and 25 ng (500 pg/mg) of metandienone to 50 mg of control hair, previously decontaminated. From this standard calibration, the precision has been evaluated.

The accuracy has not been determined because there is no qualified matrix.

Intra-day and between-day precisions for metandienone were determined using negative control hair spiked with metandienone at final concentration of 50 pg/mg ($n = 6$). This concentration has been chosen to have a average concentration of metandienone in hair according to the concentrations found in the sample.

Relative extraction recovery was determined by comparing the representative peak of metandienone extracted from negative control hair spiked at the final concentration of 50 pg/mg with the peak area of a methanolic standard at the same concentration.

The limit of detection (LOD) was evaluated with decreasing concentrations of metandienone until a response equivalent to three times the background noise was observed for the quantification transition and the other transitions. For the limit of quantification (LOQ), a response superior to 10 times the background noise is necessary.

3. Results

3.1. Validation results

The use of sodium hydroxide induced a complete digestion of the hair, but does not affect the analytes as they were found to be stable under alkaline conditions for more than 1 h at 100 °C. Actually, if the analytes were not stable at alkaline conditions, no signal would have been detected by GC–MS–MS. Sodium hydroxide is used in the majority of laboratories to digest hair.

It is desirable to produce an intense ion signal which is characteristic for the target compound. Selectivity and sensitivity are increased by almost suppressing the noise level. Selected ions and retention times of metandienone and the deuterated internal standard are reported in Table 1. The parent ion of metandienone (*m/z* 444.2) corresponds to the molecular ion; the three transitions were chosen based upon criterion of specificity and abundance.

Table 1
Selected ion (*m/z*) and retention times for metandienone and the internal standard

Analyte	Retention time (min)	Ions (*m/z*)
Metandienone	8.96	444 > 339 444 > 206 206 > 191
Nandrolone-d_3	8.50	421 > 194

The method is sensitive, specific and reproducible. A chromatogram obtained from a calibrator at 15 pg/mg is shown in Fig. 2.

The calibration curve corresponds to the linear regression between the peak area ratio of metandienone to I.S. and the final concentration of the drug in spiked hair.

Response for metandienone was linear in the range 2–500 pg/mg. The correlation coefficient was 0.9997. The precision was 10–20%. For example, with 50 mg of hair spiked with 50 pg/mg of metandienone, we determine a final concentration of 45.8 pg/mg, that is to say a difference of 10%, which is representative of the precision.

The within-batch precisions were 13.5, 14.7 and 13.4% as determined by analyzing three times six replicates (six samples were analyzed every day for 3 days, the six samples were different each day) of 50 mg of hair from the same subject and spiked with a metandienone final concentration at 50 pg/mg. The between-batch precision was 22.0% as determined by analyzing during 3 days, replicates of hair from the same subject spiked at 50 pg/mg.

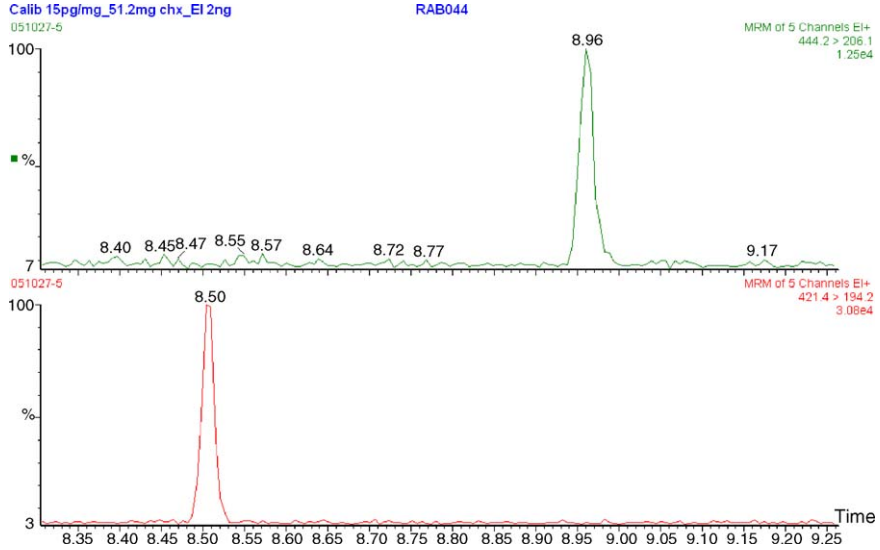

Fig. 2. Chromatogram obtained after extraction by the established procedure of a 51.2 mg hair specimen spiked for a final concentration at 15 pg/mg. Top: quantification transition *m/z* 444.2–206.1. Bottom: nandrolone-d_3 with its daughter ion at *m/z* 194.2.

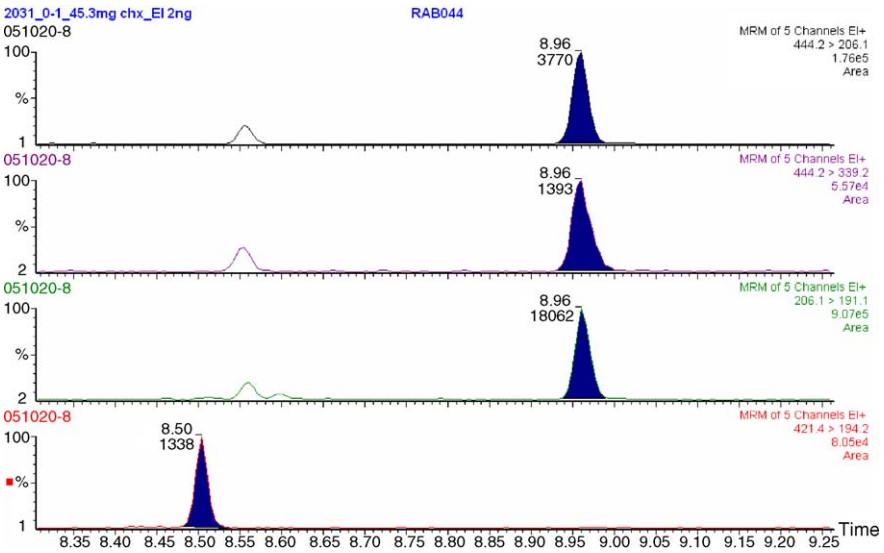

Fig. 3. Chromatogram obtained after extraction by the established procedure of a 45.3 mg hair of an athlete. Metandienone was quantified at the concentration of 78 pg/mg. Top: quantification transition for metandienone. Middle: two qualifying transitions of metandienone. Bottom: nandrolone-d_3 with its daughter ion at m/z 194.2.

The extraction recovery ($n = 3$) was determined to be 48%. The LOD of metandienone was 2 pg/mg and the LOQ was 5 pg/mg.

Extensive chromatographic procedures (two purification steps by solid-phase and liquid–liquid extractions, MS–MS) were analytical prerequisites for successful identification of metandienone in hair due to the low target concentrations.

Derivates were stable at least for 72 h (the sample have been let on the bench for 72 h. Then it was analyzed again and the same results have been obtained). Formation of the enol-TMS derivatives appears to be appropriate to obtain optimal peak shapes and intense molecular ions.

3.2. Application

The analysis of a 5 cm strand of hair, obtained from an athlete revealed the presence of metandienone at the concentrations of 78 pg/mg for the segment of 0–1 cm, 7 pg/mg for the segment of 1–2 cm, 10 pg/mg, for the segment of 2–3 cm and 108 pg/mg for the last segment (three to the end of the strand). The transition m/z 444–206 has been used to determine the concentrations of metandienone in the different segment. Fig. 3 is the chromatogram obtained for the first segment (0–1 cm) of the athlete's hair.

4. Discussion

The international literature contains very few papers dealing with the identification of metandienone in hair, actually, only one article has been written in 1999 by Gaillard et al. [2], with a limit of detection of 2.1 pg/mg. They reported one positive case for metandienone but in horse hair (15.2 pg/mg) and not in human hair. Furthermore, the method used by Gaillard et al. has an additional extraction step, in comparison with the method used by Kintz et al. or Gambelunghe et al. [9], using solid-phase extraction on NH_2 cartridges. This step is not necessary, actually, our method use only one step of solid-phase extraction on C_{18} columns and we obtain a clean extract and the same LOD.

Hair analysis is not yet been approved by the International Olympic Committee (IOC) nor the World Anti Doping Agency (WADA), but has been accepted in most Courts of Justice.

At this time, studies have shown that xenobiotic (drugs of abuse or doping agents) concentrations declined dramatically by a factor of 40–80% after cosmetic treatment, particularly after bleaching [10–12]. In consequence, it is recommended to collect pubic hair when hair is bleached. In this case, hair was bleached 15 days before sampling but it was not possible to obtain pubic or axillary hair.

This analysis was made after a positive urinalysis. Hair has been segmented to differentiate a single from a repetitive use. Each segment measured 1 cm to estimate the pattern of metandienone on a 1-month basis (at the vertex posterior, hair growth is about 1 cm/month) [7]. An urinalysis was positive one month before hair collection, the analysis of the first segment was important to discriminate a single from repetitive use. The high concentration found in the first segment does not support a single exposure to metandienone.

In spite of bleaching, the four segments were positive. The amount of metandienone in the first and the last segment was 8–10 times higher when compared with the second and the third segment. Since it concerns the hair of the same subject, it can be concluded that this increase of concentration is due to higher doses of metandienone. In the first segment, this increase is due to a partial bleaching of the segment (1/3 was not bleached). This result is consistent with a regular use of metandienone but with variable amount of drug.

In this case, hair confirms a repetitive abuse of metandienone for the athlete. The concentrations measured during analysis can be considered as underestimated due to bleaching.

5. Conclusion

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

The segmentation of hair in doping case is very important to differentiate a single from a repetitive use and like this determine if punctual doping without the knowledge of the athlete occurred or not.

It allows us to document the first positive doping case with metandienone, using hair analysis.

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