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Short communication

Doping control for methenolone using hair analysis by gas chromatography–tandem mass spectrometry

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Abstract

A sensitive, specific and reproducible method for the quantitative determination of methenolone 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 1 *M* NaOH, 15 min at 95°C, in presence of 1 ng testosterone-d_3 used as internal standard. The homogenate was neutralized and extracted using consecutively a solid-phase (Isolute C_{18} eluted with methanol) and a liquid–liquid (pentane) extraction. 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. A 1.5-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 μm film thickness) of a Hewlett-Packard (Palo Alto, CA, USA) gas chromatograph (6890 Series). Methenolone was detected by its parent ion at *m/z* 446 and daughter ions at *m/z* 208 and 195 through a Finnigan TSQ 700 MS–MS system. The assay was capable of detecting 1 pg/mg of methenolone when approximately 100 mg hair material was processed. Linearity was observed for methenolone concentrations ranging from 2 to 100 pg/mg with a correlation coefficients of 0.965–0.981. Intra-day and between-day precisions at 2, 10 and 25 pg/mg were 10.9–14.1% and 13.7–16.8%, respectively, with an extraction recovery of 97.6%. The analysis of a strand of hair obtained from two bodybuilders, revealed the presence of methenolone at the concentrations of 7.3 and 8.8 pg/mg.

Keywords: Doping control; Methenolone

1. Introduction

Traditional attempts to increase sports performance have been done by using pharmacologically active substances external to the human body. Anabolic steroids are synthetic compounds structurally related to testosterone, the male sex hormone. Methenolone or 17β-hydroxy-1β-methyl-5α-androst-1-en-3-one, seems to be less abused than the classic stanozolol or nandrolone. The drug is available in tablets as 17-acetate under the trade name Primobolan and for intra-muscular injection as 17-enanthate, under the trade name Primobolan-Depot. The drug accelerates muscle growth by an anabolic effect. Athletes can abuse methenolone because it has been claimed that it increases lean body mass, increases strength, increases aggressiveness and leads to a shorter recovery time between workouts.

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The long term misuse of anabolic androgenic steroids in human sports is still a challenge for analytical laboratories engaged in drug testing. In doping control, the anabolic steroids are commonly detected in urine by gas chromatography–mass spectrometry (GC–MS).

Abuse of anabolic steroids can be difficult to detect in urine because they are frequently taken for periods ranging from 4 to 18 weeks, alternating with drug-free periods of 1 month to 1 year [1].

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 steroid abuse [2]. 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 steroid abusers [3,4].

Hair specimens have been used for 20 years in toxicology to document chronic drug exposure in various forensic, occupationnal 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 [5].

The place of hair analysis in doping control [6] and the pharmacological criteria that are needed to document the result [7] have been described in two reviews published in 2000. Mechanisms of drug incorporation into hair and more fundamental data about the interpretation of analytical findings can be obtained in recent papers [8–10].

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

2. Materials and methods

2.1. Specimens

Hair strands were obtained from 2 bodybuilders 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 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 C_{18} 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).

Methenolone and testosterone-d_3 were purchased from Promochem (Molsheim, France).

2.3. Methenolone 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.

A 100-mg amount of decontaminated hair was incubated in 1 ml 1 *M* NaOH, 15 min at 95°C, in the presence of 1 ng of testosterone-d_3 (final concentration 10 pg/mg) used as internal standard. After cooling, the homogenate was neutralized with 1 ml 1 *M* HCl, and 2 ml of 0.2 *M* phosphate buffer (pH 7.0) was added.

The Isolute C_{18} 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 three 0.5-ml aliquots of methanol. The eluent was evaporated to dryness under a 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 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-mer-

captoethanol (1000:2:5, v/v/v), then incubated for 20 min at 60°C.

2.4. GC–MS–MS procedure

A 1.5-μl aliquot of the 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 N 55) through the column (HP5-MS capillary column, 5% phenyl–95% methylsiloxane, 30 m×0.25 mm I.D., 0.25 μm 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 100°C, maintained for 1 min, to 295°C at 30°C/min and maintained at 295°C for the final 5 min.

The detector was a Finnigan TSQ 700 operated in the electron impact and in selected reaction monitoring modes. The parent ions, *m/z* 446 and 435 for methenolone and the I.S., respectively, were selected in the first quadrupole. The corresponding daughter ions, *m/z* 208 and 195 for methenolone and 209 for the I.S., respectively, were selected in the third quadrupole after collision with argon at a cell pressure at 0.6 mTorr (1 Torr=133.322 Pa). The collision offset voltage was −5 V. The electron multiplier was operated at 1900 V.

2.5. Method validation

A standard calibration curve was obtained by adding 0.2 (2 pg/mg), 0.5 (5 pg/mg), 1 (10 pg/mg), 2.5 (25 pg/mg), 5 (50 pg/mg) and 10 (100 pg/mg) ng of methenolone to 100 mg of powdered control hair (negative for methenolone). This curve was done in triplicate. Once spiked, the powdered hair was no more decontaminated.

Within-batch and between-batch precisions for methenolone were determined using negative control hair spiked with methenolone at final concentrations of 2, 10 and 25 pg/mg (n=8).

Relative extraction recovery was determined by comparing the representative peak area of methenolone 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 limit of detection (LOD) was evaluated with decreasing concentrations of methenolone until a response equivalent to three times the background noise was observed.

3. Results

3.1. Validation results

The use of sodium hydroxide involves a complete digestion of the hair, but does not affect the analytes as they were found to be stable under alkaline conditions. However, due to the hydrolysis step, it was not possible to detect the injectable ester methenolone enantate instead of the hydrolysed parent methenolone.

Under the chromatographic conditions used, there was no interference with the analytes by any extractable endogenous materials present in hair. There were no blanc effects.

In order to obtain optimum selectivity, which is of paramount importance in doping control, the selected reaction monitoring (SRM) technique was applied. It is desirable to produce an intense ion signal which is characteristic for the target compound. Selectivity and sensitivity are extraordinarily increased by almost completely suppressing the noise level. A chromatogram obtained from a calibrator at 5 pg/mg is shown Fig. 1. Selected ions and retention times of methenolone and the deuterated internal standard are reported in Table 1. The parent ion of methenolone (*m/z* 446) corresponds to the molecular ion; the two daughter ions (*m/z* 208 and 195) were chosen based upon criterion of specificity and abundance.

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

Responses for methenolone were linear in the range 2 to 100 pg/mg. From three independent calibrations, the correlation coefficients ranged from 0.965 to 0.981.

The within-batch precisions were 14.1, 12.8 and 10.9%, as determined by analyzing eight replicates of 100 mg of hair obtained from the same subject and spiked with a methenolone final concentrations at 2, 10 and 25 pg/mg, respectively. The between-

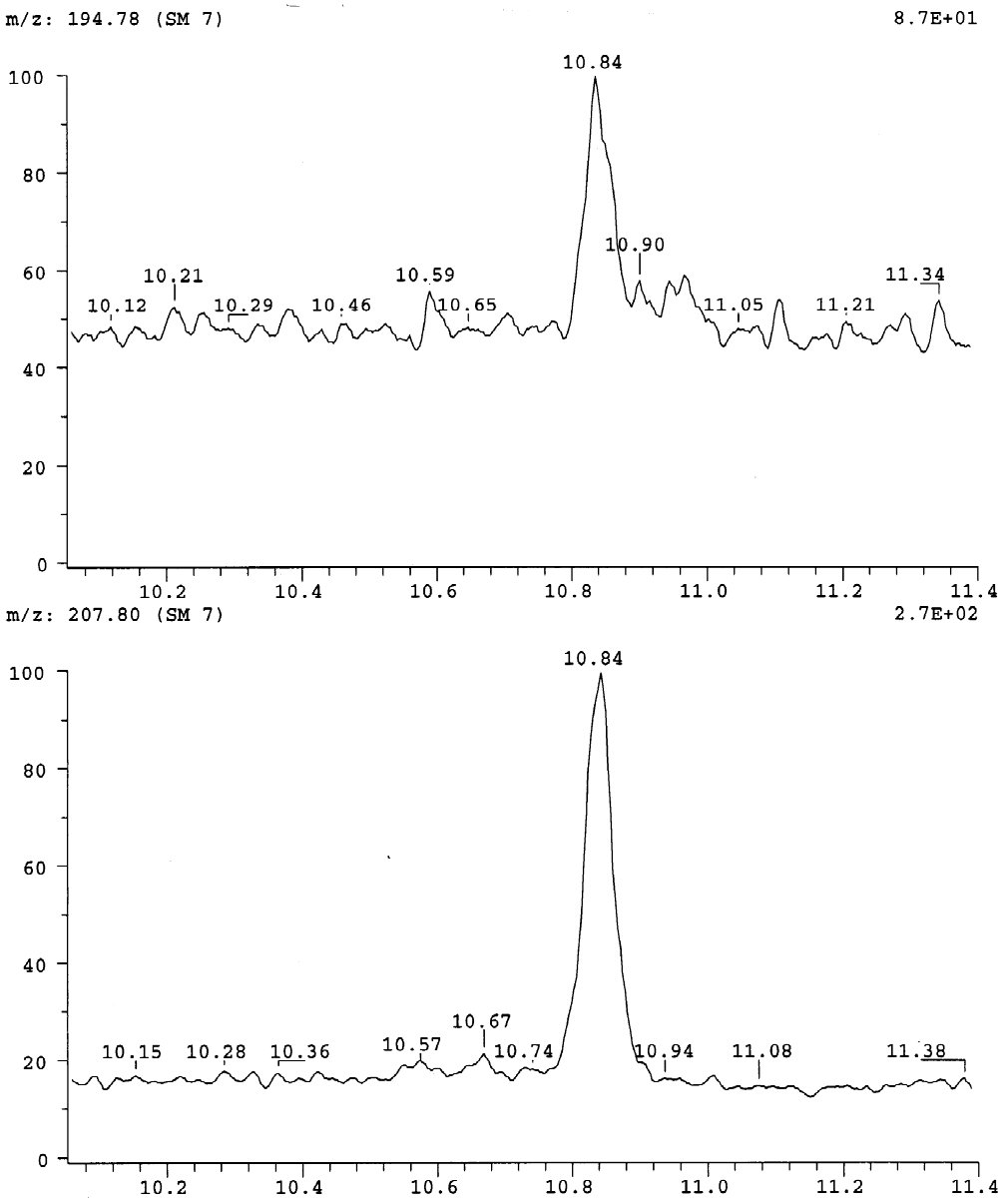

Fig. 1. SRM chromatogram obtained after extraction by the established procedure of a 101-mg hair specimen spiked for a final methenolone concentration at 5 pg/mg. Top: First daughter ion at *m*/*z* 195 and bottom: second daughter ion at *m*/*z* 208.

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

Analyte	Retention time (min)	Ions (*m*/*z*)
Methenolone	10.84	446 to 208 and 446 to 195
Testosterone-d_3	10.54	435 to 209

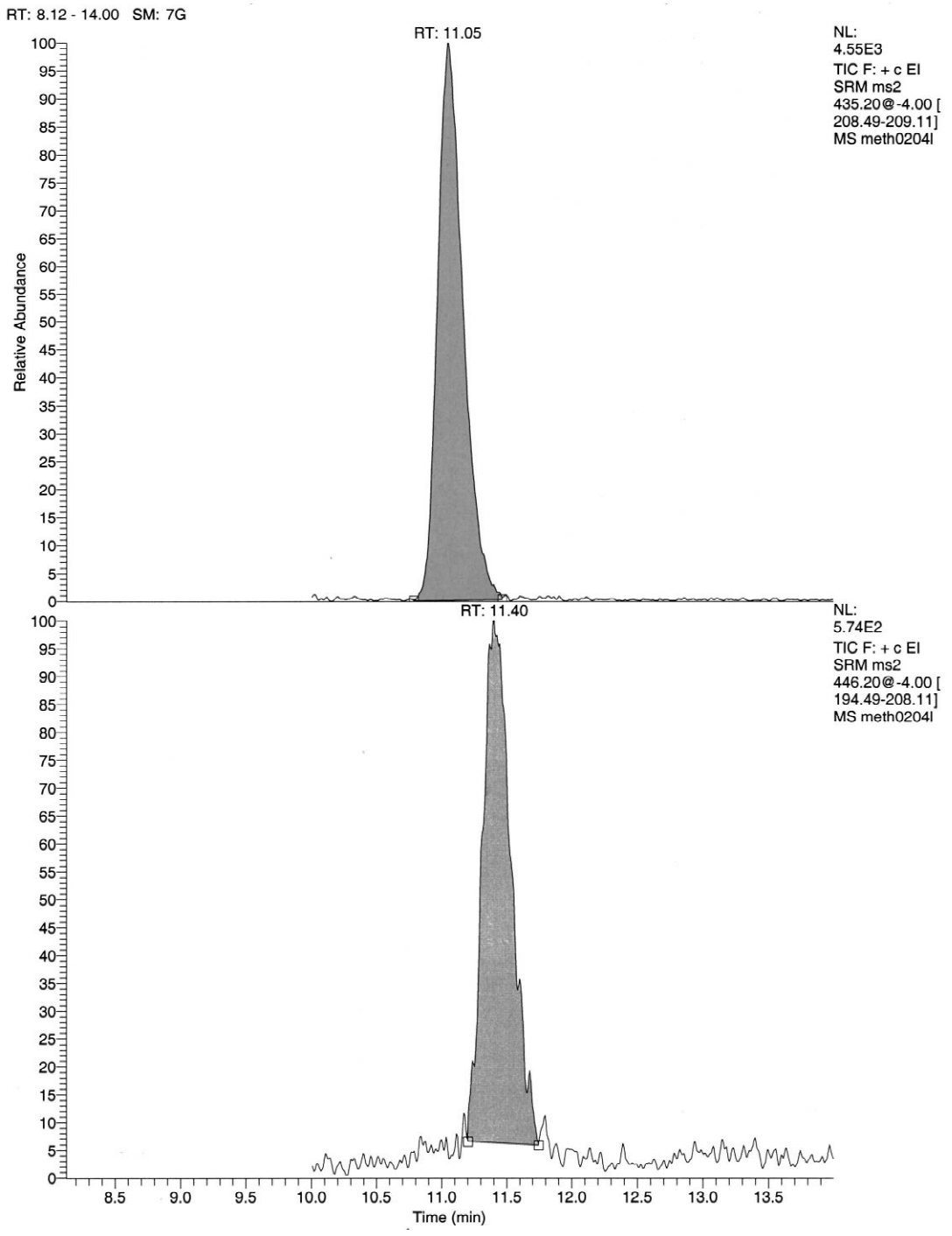

Fig. 2. SRM chromatogram obtained after extraction by the established procedure of a 101-mg hair specimen of a bodybuilder. Methenolone was quantified at the concentration of 7.3 pg/mg. Top: testosterone-d_3 with its daughter ion at m/z 209 and bottom: methenolone with the sum of its two daughter ions at m/z 195 and 208.

batch precisions were 16.8, 15.9 and 13.7%, as determined by analyzing during 8 consecutive days, replicates of hair from the same subject spiked at 2, 10 and 25 pg/mg, respectively.

The extraction recovery ($n=3$) was determined to be 97.6%. The LOD of methenolone was 1 pg/mg. The limit of quantitation was the first point of the calibration curve, that is 2 pg/mg.

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

Derivatives were stable at least for 72 h. 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 strand of hair, obtained from two bodybuilders revealed the presence of methenolone at the concentrations of 7.3 and 8.8 pg/mg. Fig. 2 is the chromatogram obtained in SRM mode of the first athlete. These results were not challenged by the subjects.

4. Discussion

The international literature is very poor in papers dealing with the identification of methenolone in hair. In 1999, Deng et al. [11] tested anabolic steroids in head hair from seven male steroid abusers. However, with an LOD at 20 pg/mg and a recovery at 24%, the authors failed to identify any positive. Thieme et al. [12] detected the ester methenolone enantate in the hair of a bodybuilder, using tandem MS.

As proposed by these previous papers, the hair preparation for methenolone investigation involved strong alkaline hydrolysis in order to enhance the recovery of the drug. Once hydrolyzed in the, aqueous medium, the hair homogenates were extracted exactly according to the International Olympic Committee (IOC) proposed technique for urine that was found suitable for hair in this laboratory [5,7].

When using hair in a suspected doping case, particularly when urine of the athlete was positive and hair negative, 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 and should not overrule a positive urine result. Nevertheless, the negative hair findings lends enough ambiguity to the positive urine result, coupled with the sporting consequences for the athlete, that substantial justice refereeing occurs [7].

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.

Hair can confirm repetitive abuse; however, some issues have to be discussed before considering hair as a valid specimen by the IOC and the International Sport Federations. According to the French law, hair is now considered as a valid specimen to document doping practices [13].

5. Conclusion

The sensitive, specific and reproducible method developed seems to be suitable for the detection and quantification of methenolone in human hair. The estimated target concentrations are below 25 pg/mg.

Hair analysis may be a useful adjunct to conventional drug testing in sports. It should not be considered as an alternative to urinalysis, but as a complement. This technology may find useful applications in doping control, if accepted by the International Olympic Committee, but the definition of legally defensible cut-off values for doping would require much more data.

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