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Testing of the anabolic stanozolol in human hair by gas chromatography–negative ion chemical ionization mass spectrometry

V. Cirimele*, P. Kintz, B. Ludes

Institut de Médecine Légale, 11 Rue Humann, 67085 Strasbourg, France

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Abstract

A sensitive, specific and reproducible method for the quantitative determination of stanozolol in human hair has been developed. The sample preparation involved a decontamination step of the hair with methylene chloride and the sonication in methanol of 100 mg of powdered hair for 2 h. After elimination of the solvent, the hair sample was solubilized in 1 ml 1 *M* NaOH, 15 min at 95°C, in the presence of 10 ng stanozolol-d_3 used as internal standard. The homogenate was neutralized and extracted using consecutively a solid-phase (Isolute C_{18}) and a liquid–liquid (pentane) extraction. After evaporation of the final organic phase, the dry extract was derivatized using 40 μl MBHFA–TMSI (1000:20, v/v), incubated for 5 min at 80°C, followed by 10 μl of MBHFBA, incubated for 30 min at 80°C. The derivatized extract was analyzed by a Hewlett-Packard GC–MS system with a 5989 B Engine operating in the negative chemical ionization mode of detection. Linearity of the detector response was observed for stanozolol concentrations ranging from 5 to 200 pg/mg with a correlation coefficient of 0.998. The assay was capable of detecting 2 pg of stanozolol per mg of hair when approximately 100 mg hair material was processed, with a quantification limit set at 5 pg/mg. Intra-day precision was 5.9% at 50 pg/mg and 7.8% at 25 pg/mg with extraction recoveries of 79.8 and 75.1%, respectively. The analysis of a 3-cm long hair strand, obtained from a young bodybuilder (27 year old) assuming to be a regular user of Winstrol (stanozolol, 2 mg), revealed the presence of stanozolol at the concentration of 15 pg/mg.

Keywords: Anabolic steroids; Stanozolol

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.

Stanozolol, 17α-methyl-17β-hydroxy-5α-androstano-(3,2-C)-pyrazole, has been one of the most abused anabolic steroids. For example, stanozolol was among the anabolic steroid-positive tests reported at the 1988 Olympic Games [2].

The standard of testing for anabolic steroids for doping control is gas chromatography coupled to mass spectrometry (GC–MS) conducted on an urine

*Corresponding author. Tel.: +33-3-8835-8725; fax: +33-3-8824-0085.

E-mail address: vincent.cirimele@medecine.u-strasbg.fr (V. Cirimele)

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sample, and performed in accredited laboratories. However, stanozolol has poor gas chromatographic behavior and the measured concentrations are generally low due to its slow excretion rate (only 16% during the first day) [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, alternating with drug-free periods of 1 month to 1 year [4].

Long-term effects (severe cardiovascular 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 [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 [6–10].

Hair specimens have been used for 20 years in toxicology to document chronic drug exposure in various forensic, occupational and clinical situations [11]. The major practical advantage of hair testing compared with urine testing for drugs is that it has a larger surveillance window: weeks to months, depending on the length of the hair shaft against 2–4 days for most xenobiotics, with the exception of anabolic steroids in an ester preparation such as testosterone enantate or nandrolone decanoate. However, stanozolol is not abused in an ester preparation. 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.

There are few papers in the international literature dealing with the identification of stanozolol in hair. One paper focused on rats receiving 20 mg/kg stanozolol for 3 days [12]. However, the growing interest of scientists in the detection of anabolic drugs was observed in late 1998 [13–18]. At this time, no controlled studies involving stanozolol administration in humans and simultaneous analysis in both plasma and hair, have been published.

This paper describes a very sensitive and specific analytical method for the detection and quantification of stanozolol in human hair by GC–MS in the negative chemical ionization (NCI) mode of detection.

2. Materials and methods

2.1. Specimen

Hair strand was obtained from a young bodybuilder (27 year old) who declared to be a regular user of Winstrol (stanozolol, 2 mg). Full-length hair strands, which were approximately 3-cm long, were taken at the surface of the skin from the vertex and stored in plastic tubes at room temperature.

Control 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.

Stanozolol, 3′OH-stanozolol, 16β-OH-stanozolol and stanozolol-d_3 were purchased from Radian (Austin, TX, USA).

Isolute C_{18} columns were purchased from Touzart et Matignon (Courtaboeuf, France).

N-Methyl-*N*-trimethylsilyl-heptafluorobutyramide (MSHFBA), and trimethylsilylimidazole (TMSI) were purchased from Fluka (Buchs, Switzerland). *N*-Methyl-bis-heptafluorobutyramide (MBHFBA) was purchased from Macherey–Nagel (Düren, Germany).

2.3. Stanozolol extraction

Before the extraction step, the hair specimens (obtained from the bodybuilder and from the laboratory personal) were 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 powdered hair was sonicated for 2 h in 5 ml of methanol. After centrifugation of the homogenate, the methanolic phase was removed and the powdered hair incubated in 1 ml 1 *M* NaOH for 15 min at 95°C, in the presence of 10 ng of

stanozolol-d_3 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) were 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 aliquots of 0.5 ml of methanol. The eluent 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 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 40 μl MBHFA–TMSI (1000:20, v/v), incubated for 5 min at 80°C. After cooling, 10 μl of MBHFBA were added and the sample incubated again for 30 min at 80°C.

2.4. GC–MS procedure

A 1.5-μl aliquot of the derivatized extract was directly injected into the column of a Hewlett-Packard (Palo Alto, CA, USA) gas chromatograph (5890 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 μm film thickness) was 1.0 ml/min. The injector temperature was 240°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°C, maintained for 1 min, to 295°C at 30°C/min and maintained at 295°C for the final 7 min.

The detector was a Hewlett-Packard 5989 B Engine operated in the NCI mode of detection. Methane was used as reagent gas at an apparent pressure of 1.4 Torr in the ion source (1 Torr= 133.322 Pa). Ion source and quadrupole temperatures were of 200°C and 100°C, respectively. The electron multiplier voltage was set at +600 V above the NCI-tune voltage. Acquisition was made in the single ion monitoring (SIM) mode.

2.5. Method validation

A standard calibration curve was obtained by adding 0.5 (5 pg/mg), 2 (20 pg/mg), 5 (50 pg/mg), 10 (100 pg/mg), 15 (150 pg/mg), and 20 (200 pg/mg) ng of stanozolol to 100 mg of powdered control hair (negative for stanozolol).

Within-run precision for stanozolol was determined using negative control hair spiked with stanozolol at the final concentrations of 25 and 50 pg/mg (n=8).

Relative extraction recovery was determined by comparing the representative peak area of stanozolol extracted from negative control hair spiked at the final concentrations of 25 and 50 pg/mg with the peak area of a methanolic standard at the same concentration.

The detection limit (LOD) was evaluated with decreasing concentrations of stanozolol until a response equivalent to three-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. Complete dissolution of the hair matrix was chosen as stanozolol is stable in 1 *M* NaOH at 95°C for at least 30 min. This hydrolysis step enhances the extraction recovery of the low concentrated stanozolol in human hair. The negative chemical ionization spectra of stanozolol, 3′OH- and 16βOH-stanozolol are shown in Figs. 1–3, respectively. Selected ions and retention times of stanozolol, its metabolites 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.

The GC–NCI-MS analysis of the hair extract shows linear behavior within the concentration range from 5 to 200 pg/mg, with a correlation coefficient of 0.998. The calibration curve corresponds to the linear regression between the peak-area ratio of drug to internal standard and the final concentration of

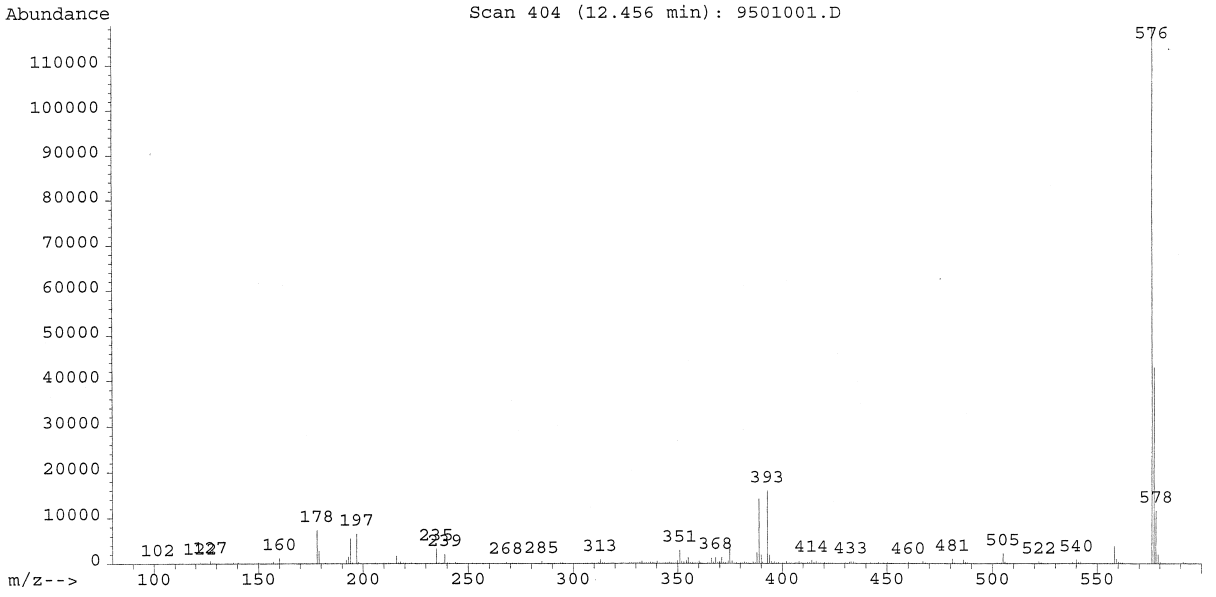

Fig. 1. Mass spectra of HFB-derivative of stanozolol in negative chemical ionization mode of detection with methane.

stanozolol in spiked hair. The equation of the curve was $y=37.821x-7.682$.

The LOD of stanozolol, determined for a signal-to-noise ratio of 3, was 2 pg/mg. Quantification limit of the established method was determined by the lower concentration value of the calibration curve, which is 5 pg/mg.

The within-run precision values were 5.9% and

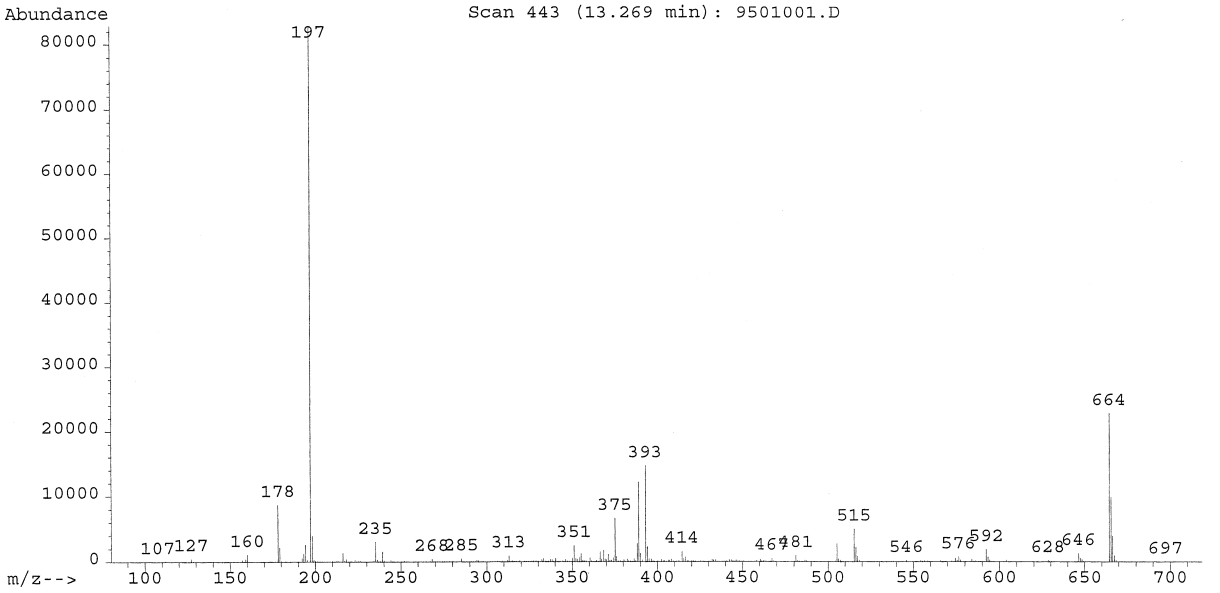

Fig. 2. Mass spectra of 3′OH-stanozolol derivative in NCI mode.

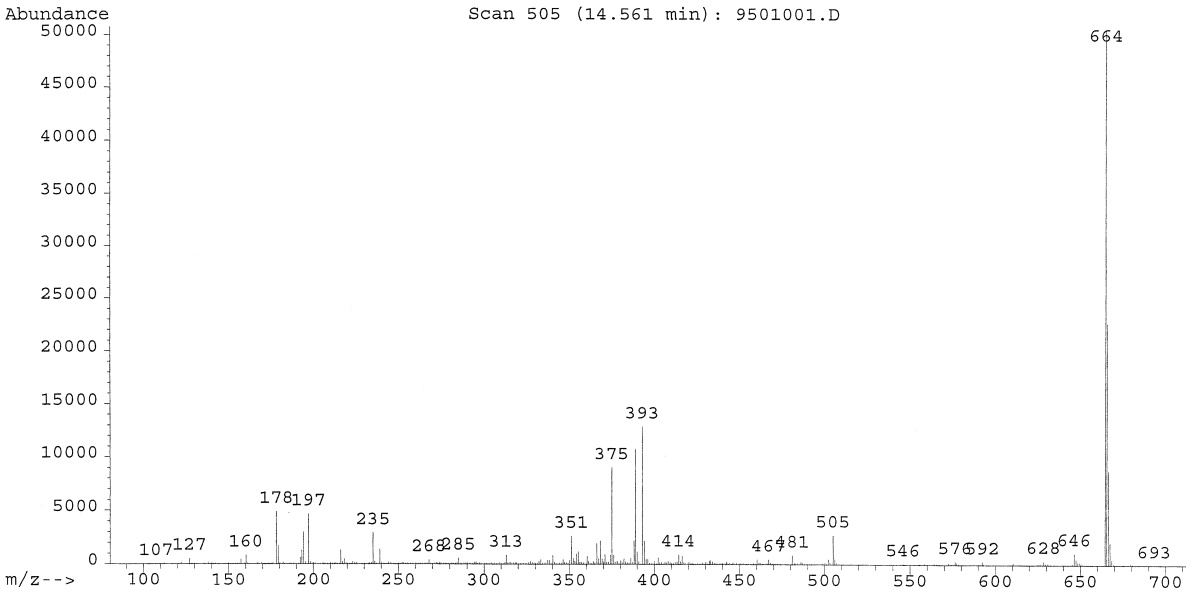

Fig. 3. Mass spectra of 16βOH-stanozolol derivative in NCI mode.

7.8%, as determined by analyzing eight pulverized replicates of 100 mg of control hair spiked at 50 pg/mg and 25 ng/mg, respectively. The extraction recovery ($n=8$) was determined to be 79.8% at the final stanozolol concentration of 50 pg/mg and 75.1% at 25 pg/mg.

3.2. Application

The analysis of a hair strand, obtained from a young bodybuider (27 year old), revealed the presence of stanozolol at a concentration of 15 pg/mg (Fig. 4). Its main urinary metabolite, 3′OH-stanozolol, was not detected in hair. This finding was in accordance with his declaration concerning the chronic consumption of stanozolol.

Table 1
Selected ion (m/z) and retention times for each analyte[a]

Analyte	Retention time (min)	Ions (m/z)
Stanozolol	12.44	197–393–576
Stanozolol-d_3	12.42	579
3′OH-Stanozolol	13.27	197–393–664
16βOH-Stanozolol	14.56	197–393–664

[a] The underlined ions were used for quantification.

3.3. Discussion

During the initial steps of the method development, various extraction procedures were tested. Direct hydrolysis in NaOH was unsuccessful for the identification of stanozolol at low concentration due to a high background noise from endogenous steroids. This was solved by removing them in methanol. The preliminary methanolic purification step, first described by Thieme et al. [19], was necessary to eliminate all endogenous compound present in hair and interfering with stanozolol. The SIM chromatograms obtained have less background noise and interfering peaks than those we previously published as a general screening procedure for anabolic steroids in human hair [13]. As described by Thieme et al. [19], it was also observed that stanozolol is not extracted in the first methanol fraction, probably due to its basic properties. Stanozolol seems to be linked to hair matrix by specific and strong interactions which exclude direct solubilization by solvents. Classic derivatization agents leading to silylation such as MSTFA were not suitable for negative chemical ionization, as they did not produce any electron attractive ions. As bench-top GC–MS in the electron impact mode was not sensitive enough, the

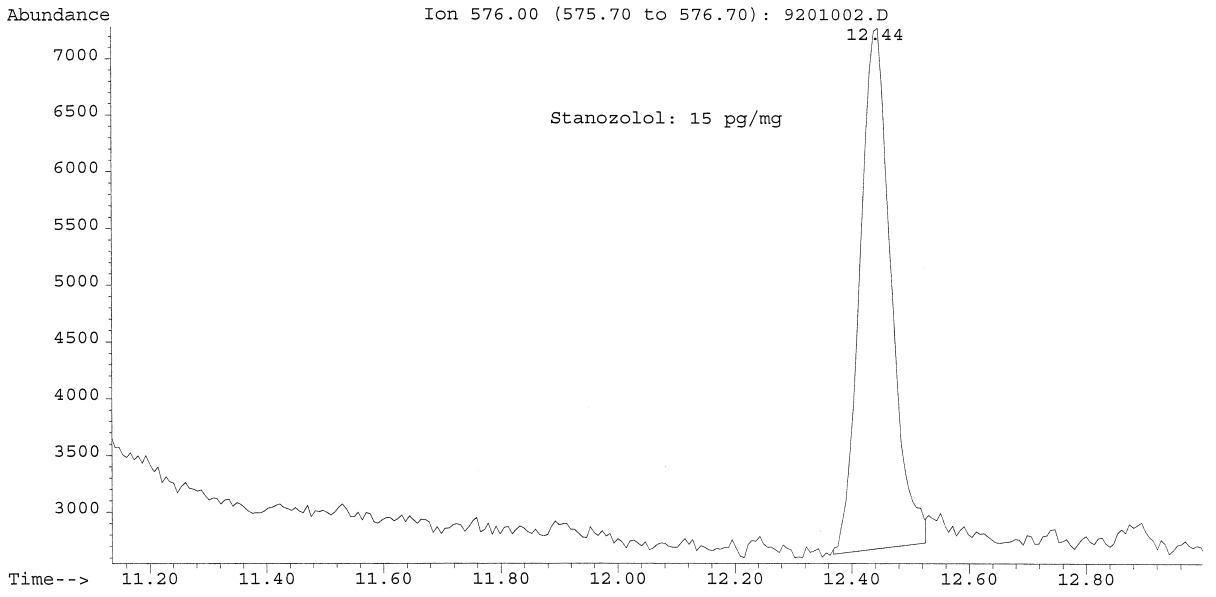

Fig. 4. SIM chromatogram obtained after extraction by the established procedure of a 104-mg hair specimen of a bodybuilder. Stanozolol was identified and quantified at a concentration of 15 pg/mg.

use of negative chemical ionization mode became mandatory.

Extensive chromatographic procedures (three purification steps, methanolic incubation, solid-phase extraction and liquid–liquid extraction, combined with sophisticated derivatization procedure and negative chemical ionization mode of detection) were analytical prerequisites for successful identification of stanozolol in hair due to the low concentration.

The determined concentration (15 pg/mg) is largely lower than those determined by Höld et al. [12] in the hair of rats (40.2 to 751.4 pg/mg). This was certainly due to the high dose administered to the rats (20 mg/kg intraperitoneally once daily for three days). Our result is also largely lower than those determined by Thieme et al. [19] in a hair sample obtained from a bodybuilder (stanozolol: 180 pg/mg, 3′OH-stanozolol: 6 pg/mg) and lower than those reported by Kintz et al. [13] for two bodybuilders (135 and 156 pg/mg). This can explain why 3′OH-stanozolol, the primary stanozolol metabolite, was not detected in this case. As it is generally the case with hair analysis, the parent drug is found in higher concentration than the metabolites. In the case reported by Thieme et al. [19], the ratio parent drug-to-metabolite was 30:1.

4. Conclusion

The sensitive, specific and reproducible method developed seems to be suitable for the detection and quantification of stanozolol in human hair. HFB-derivative of stanozolol in the negative chemical ionization mode of detection represents the technique of choice to test stanozolol in hair regarding the low concentration determined.

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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