CONFIRMATION OF LONG TERM EXCRETED METABOLITES OF METANDIENONE BY GAS CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY

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Metoda folosită de laboratoarele de control doping pentru dovedirea abuzului de Metandienonă în sport se bazează pe detecţia metaboliţilor săi urinari de remanenţă îndelungată 17β-metil-5β-androst-1-ene-3α,17α-diol şi 17β-hidroximetil,17α-metil-18-norandrost-1,4,13-trien-3-onă. Dacă în urma unei analize de testare iniţială cu extracţie lichid-lichid, o probă de urină supusă controlului doping este declarată suspectă pe Metandienonă este necesară analiza de confirmare prin care metaboliţii de remanenţă îndelungată ai acestei substanţe sunt izolaţi prin extracţie solid-lichid urmată de îndepărtarea fracţiei libere şi extracţie cu n-pentan şi identificaţi prin cromatografie de gaze cuplată cu spectrometrie de masă de joasă rezoluţie. Îndeplinirea criteriilor de confirmare specifice pentru mai mulţi metaboliţi ai aceluiaşi compus, oferă dovezi suplimentare în sprijinul unei decizii corecte în controlul doping.

The method used by doping control laboratories to prove Metandienone abuse in sport is based on the detection of its urinary long-term metabolites 17β-methyl-5β-androst-1-ene-3α,17α-diol and 17β-hydroxymethyl,17α-metil-18-norandrost-1,4,13-trien-3-one. If a urine sample is declared suspect for Metandienone abuse after an initial test analysis with liquid-liquid extraction, it is necessary a confirmation analysis for the long-term metabolites that are isolated by solid-liquid extraction followed by removal free fraction and extraction with n-pentane and identified by gas chromatography coupled with low resolution mass spectrometry. By meeting the specific identification criteria for more than one metabolite of the same parent compound, additional evidence could be obtained in the decision making process in doping control.

Keywords: Methandienone, confirmation, GC/MS

1. Introduction

The use of anabolic androgenic steroids in sport is prohibited by World Anti-Doping Agency's (WADA) requirements [1]. The abuse of this class of

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substances is controlled by analyzing the urine samples collected from the athletes, both in and out of competition, by gas chromatography coupled with mass spectrometry technique (GC/MS). Metandienone (17α-methyl-androsta-1,4-dien-17β-ol-3-one) was reported as the first anabolic androgenous steroid used by the athletes [2], available as tablets with different names as early 1956. Pharmaceutical products containing this substance accelerate muscle growth by anabolic effects and lead to a shorter recovery between training. The Metandienone metabolism was studied intensely and involves many stages: hydrogenation of double bond between C_4 and C_5 by 5β-reductase, reduction of ceto group of C_3 atom by 3α-dehydrogenises and hydrogenation of double bond between C_1 and C_2. Some studies [3-9] showed that the metabolizing results in many compounds that can be used to establish the abuse of Metandienone (Fig. 1). Thus, the researches were focused on confirmation of main Metandienone metabolites detectable in athletes urine, 17α-methyl-5β-androstane-3α,17β-diol (**2**), 17β-methyl-5β-androst-1-ene-3α,17α-diol (**3**), 18-nor-17,17-dimethyl-5β-androst-1,13-dien-3α-ol (**4**) and 17β-hydroxymethyl,17α-methyl-18-norandrost-1,4,13-trien-3-one (**5**), excreted as conjugated fraction. As target analytes for the long-term detection of Methandienone abuse were selected 17β-methyl-5β-androst-1-ene-3α,17α-diol (**3**) and 17β-hydroxymethyl,17α-methyl-18-norandrost-1,4,13-trien-3-one (**5**) [10,11].

Following the acceptance criteria established by WADA, a prohibited substance is confirmed by comparing it with a reference material, analyzed in parallel with the suspected sample, while the concentration domains in both sample and reference need to be similar [12]. Technical document TD2010IDCR of International Standard for Laboratories ver.6.0/2009 [13] states that a prohibited substance may be reported as present in a suspected sample if the retention time of the analyte in the chromatographic column does not differ with more than 2 (two) percent or ± 0,1 minutes (whichever is smaller) in comparison with a sample containing the reference substance, while the relative abundances of three diagnosis ions do not differ with more than the figure indicated in Table 1, in comparison with the relative intensities of the same ions obtained from a reference urine and signal/noise ratio of the least intense diagnosis ion to be greater than 3:1 (three to one).

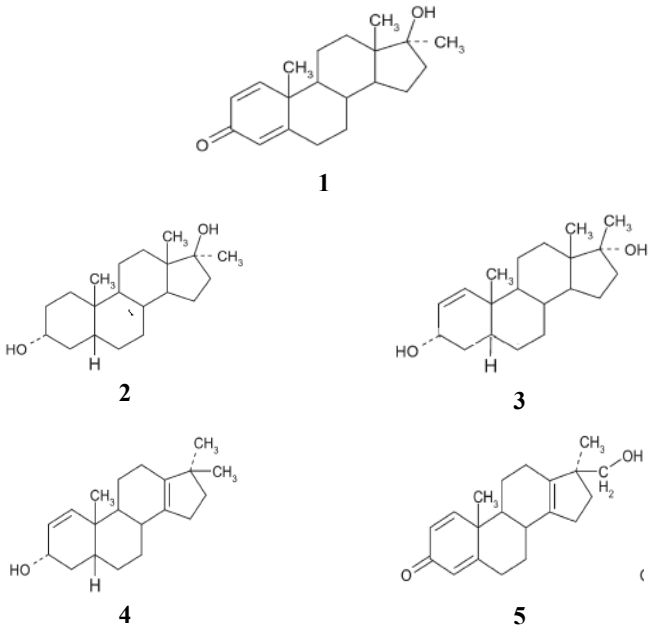


Fig. 1. Metabolism of Metandienone (**1**): 17α-methyl-5β-androstane-3α,17β-diol (**2**), 17β-methyl-5β-androst-1-ene-3α,17α-diol (**3**), 18-nor-17,17-dimethyl-5β-androst-1,13-dien-3α-ol (**4**) and 17β-hydroxymethyl,17α-methyl-18-norandrost-1,4,13-trien-3-one (**5**)

Table 1

Maximum Tolerance Windows for Relative Ion Intensities to Ensure Appropriate Confidence in Identification

Relative Abundance (% of base peak)	EI-GC/MS; CI-GC/MS; GC/MS^n; LC/MS; LC/MS^n
>50%	±10% (absolute)
25% - 50%	±20% (relative)
5% - 25%	±5% (absolute)
<5%	±50% (absolute)

2. Experimetal

Materials

The reference substances for Metandienone metabolites and internal standard, methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one) were purchased from National Measurement Institute (Germany). Primary stock solutions (1mg/mL) were prepared in a methanol and stored at <0°C, protected from light. Intermediate working solutions were obtained through a ten-fold dilution of these primary stock solutions. Derivatization agent N-methyl-N-

trimethylsilyltrifluoroacetamide MSTFA (for gas chromatography) was produced by Alfa Aesar GmbH&CohG and the enzyme β-Glucuronidase from E.coli was from Roche Diagnostics Manheim. All the other reagents and solvents were of analytical and chromatographic grade and were purchased from Sigma and Merck.

A urine sample obtained after orally administration of a single dose of Metandienone (25mg Dianabol) to a healthy male volunteer (30 years) was used for the confirmation analysis. As a human subject was involved, for this study an ethical approval was obtained from the local ethical commission.

Isolation of Metandienone metabolites from urine by liquid-liquid extraction

Urine samples were prepared for regular doping control analysis adopted after Schänzer and Dönike [14] and implemented in Romanian Doping Control Laboratory according to the internal laboratory standard operating procedures in order to detect anabolic agents. In order to identify the Methandienone metabolites, internal standard (methyltestosterone, 500ng/mL), 1mL phosphate buffer (pH 7) and 25μL of β-glucuronidase from E.Coli solution were added to 2mL of urine. The samples were hydrolyzed during 90 minutes at 50°C. After adjusting the pH to approximately 9 with potassium carbonate the analytes were extracted with tert-butyl-methyl-ether. The samples were shaked mechanically 15 minutes (100oscilations/min) and were centrifuged for 15 minutes at 2500rpm and the organic phase was separated and evaporated to dryness under nitrogen stream at 40^0C. The samples were dried in a desiccator for 60 minutes and for GC/MS analysis, the dry residues were derivatized using 100μL MSTFA/NH4I/ethanethiol (1000/2/3; v/w/v) at 60°C for 30 minutes and 3μL were injected into the gas chromatography/mass spectrometry (GC/MS) system.

Isolation of Metandienone metabolites from urine by solid-liquid extraction

In order to confirm the abuse of Metandienone, the suspicious urine sample was extracted by solid-liquid extraction (SLE) on Amberlite XAD_2 polystyrene resin with application of additional steps of purification, separation of free fraction and extraction in n-pentane of interest compounds. 2mL of urine sample were submitted to the extraction methodology for the the androgenous anabolic steroids in the conjugated fraction: column extraction containing Amberlit XAD_2 resin activated previously; elution of the interest compounds from the column with 2mL methanol; extraction with tert-butyl-methyl-ether; separation of the ether layer (free fraction); enzymatic hydrolysis with β-glucuronidase from E.Coli; extraction with n-pentane; evaporation and drying of the residue in vacuum; derivatization reaction.

Together with the sample, were extracted negative control samples and positive control samples fortified with interest metabolites in concentrations close to those estimated in initial testing, under the same conditions and analyzed by GC/MS. All the samples contained 500ng/mL internal standard methyltestosterone.

GC/MS analysis

The compounds resulting from the derivate reaction were identified through gas chromatography coupled with mass spectrometry using the system GC/MS Agilent Techologies 6890N/5973. 3μL derivatized extract were injected split 1:10 on a capillary column UTRA 1 J&W Scientific (dimethylpolisiloxan cross-linked stationary phase 100%, length 17m, interior diameter dimension 0,2mm and stationary phase layer thickness 0,11μm) on a constant flow rate of helium carrier gas of 1ml/min. The oven temperature program was as follows: 160°C (2 min), 5°C/min→ 255°C (0 min), 30°C/min → 285°C (5 min) and 60°C/min →300°C (3.75 min). The electron energy was set at 70eV and the ion source temperature was set at 230°C.

The acquisition was performed in the SIM mode (selected ion monitoring) by recording two groups of seven (m/z 143, 216, 358, 448, 133, 339, 442), respectively two (m/z 301, 446) mass fragments characteristic for the interest metabolites and internal standard.

3. Resuls and discussions

In this study we intended to describe the initial testing and confirmation procedures for Metandienone abuse. Thus, in order to monitor the metabolites of Metandienone, we extracted a sample resulted from an excretion study performed after oral administration of 25mg Metandienone, by liquid-liquid extraction (LLE) and we analyzed it by GC/MS. Out of all Metandienone metabolites (Fig. 1), metabolites **3** and **5** show a particular interest in doping control analysis because they fall out of the body slower, therefore they remain in the body for a longer period of time.

Fig. 2 shows the ion chromatogram and mass spectrum for metabolites **3** and **5**. By the use of the GC/MS method, the monitored compounds were detected at t_R 12.40 min (**3**) and t_R 15.15 min (**5**). At retention time corresponding to chromatographic elution of **3** metabolite, the sample shows less intense and not very well shaped peaks, but the mass spectrometer contains this metabolite's characteristic ions m/z 143, 216, 358, 448, while the signals of metabolite **5** characteristic ions are very weak and the mass spectrum shows the presence of other masses with intensities closed to those of m/z 133, 339, 442 characteristic ions. Under these circumstances, the sample may be declared suspect for abuse of

Metandienone and can be extracted for the confirmation procedure by SLE extraction and additional purification steps.

In GC/MS, the elevated background noise observed after LLE can be reduced through a procedure of purification via Amberlite XAD_2 polystyrene resin and separation of the free fraction. After XAD_2 purification step, the removal of free fraction, the extraction of the interest compound with n-pentane and GC/MS analysis, it was observed a better eluation profile. It should be noticed that the target metabolites are very well distinguished and exhibit clear and symmetrical peaks. Fig. 3 depicts chromatograms generated from the suspicious sample for metabolites **3** and **5**.

In Fig. 4 chromatograms generated from a blank urine sample spiked at 50ng/mL of metabolites **3** and **5** are shown. It should be noticed that no interferences from matrix exist at the retention time values characteristic for each metabolite.

Table 2 and 3 show the compliance between the relative abundances of the transitions and the retention times of the suspicious sample and the 50ng/mL reference and the concentration estimation data for the interest metabolites. Retention times (t_R) and signals height were obtained from chromatograms of the suspicious sample (Fig. 3) and reference urine spiked at 50ng of each metabolite/mL (Fig. 4). The concentrations levels of the metabolites (4,83ng/mL **3** and 14,80ng/mL **5**) were estimated by direct comparison of response factors of the base peaks (m/z 143, respectively 133) against the internal standard, in both reference and suspicious sample. For confirmation of Metandienone abuse, criteria of identification requested by WADA [13] were applied, using Excel spreadsheets. The relative ion abundance of at least three diagnostic ions should fulfill the acceptable ranges presented in technical document TD2010IDCR and the relative retention time should not differ by more than ± 1% from that of the same substance in reference urine. By introducing the signals height into the Excel, one may notice that their relative abundances are closed to the ones of the reference and they fit the limits of acceptance domain for all the chosen fragments. At the same time, the confirmation criteria for retention time are met. Taking into account that the confirmation criteria for both long term metabolites of Metandienone are met, the sample can be reported as adverse analytical finding for this anabolic steroid.

Table 2

The criteria for the identification of 17β-methyl-5β-androst-1-ene-3α,17α-diol (3)

Reference sample- 50ng/mL (3)					**Sample**		
T_R (min)	m/z	Signal`s height	Relative abundence (%)	Acceptance range (%)	Relative abundentce (%)	Signal`s height	T_R (min)
12.46	143	92404	100.0		100.0	10640	12.41
T_R-SI	216	14420	15.6	10.6-20.6	17.9	1905	T_R-SI
16.72	358	10595	11.5	6.5-16.5	12.7	1347	16.63
T_{RR}	448	2432	2.6	1.3-3.9	2.9	308	T_{RR}
0.745							0.746
Internal standard: MT							$\%T_{RR}$
Reference sample - 50ng/mL (3)			339710				0.14%
Sample			404972				$\%T_R$ - 0.40%
Concentration of (3)							ΔT_R - 0.050'
Reference sample:			50	ng/ml			
Sample:			4.83	ng/ml			

Table 3

The criteria for the identification of 17β-hydroxymethyl,17α-methyl-18-norandrost-1,4,13-trien-3-one (5)

Reference sample - 50ng/mL (5)					**Sample**		
T_R (min)	m/z	Signal`s height	Relative abundence (%)	Acceptance range (%)	Relative abundence (%)	Signal`s height	T_R (min)
15.23	133	10746	100.0		100.0	3791	15.17
T_R-SI	339	5099	47.5	38-56.9	45.3	1719	T_R-SI
16.72	442	2003	18.6	13.6-23.6	16.3	619	16.63
T_{RR}							T_{RR}
0.910							0.912
Internal standard: MT							$\%T_{RR}$
Reference sample - 50ng/mL (5)			339710				0.15%
Sample			404972				$\%T_R$ - 0.39%
Concentration of (5)							ΔT_R - 0.060'
Reference sample:			50	ng/ml			
Sample:			14.80	ng/ml			

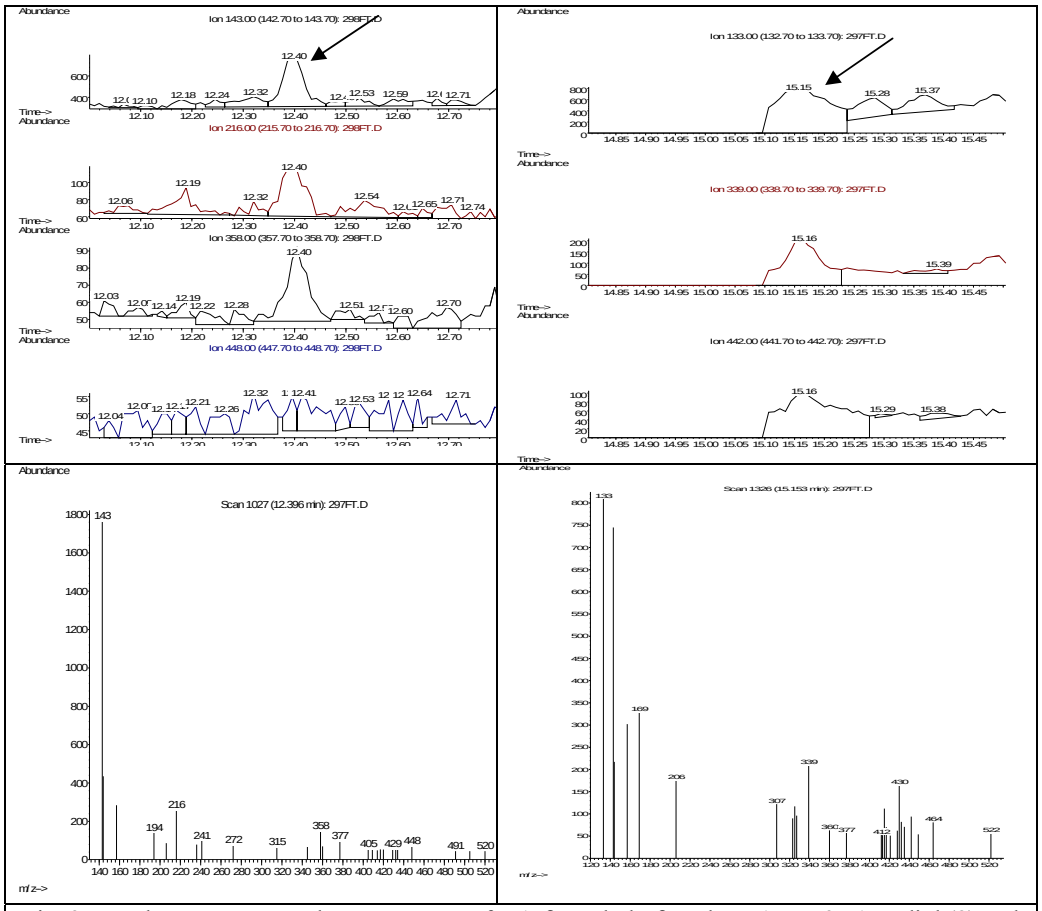
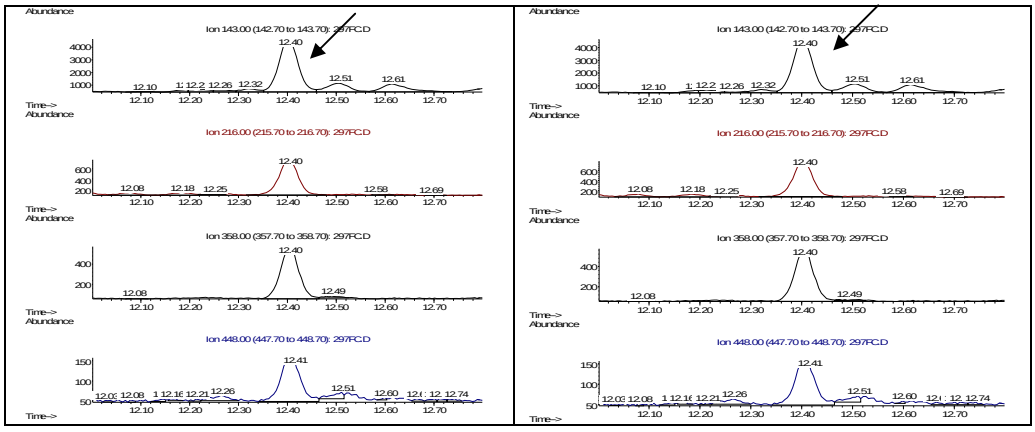


Fig. 2. Ion chromatogram and mass spectrum for 17β-methyl-5β-androst-1-ene-3α,17α-diol (**3**) and 17β-hydroxymethyl,17α-methyl-18-norandrost-1,4,13-trien-3-one (**5**) in the suspicious sample after liquid-liquid extraction



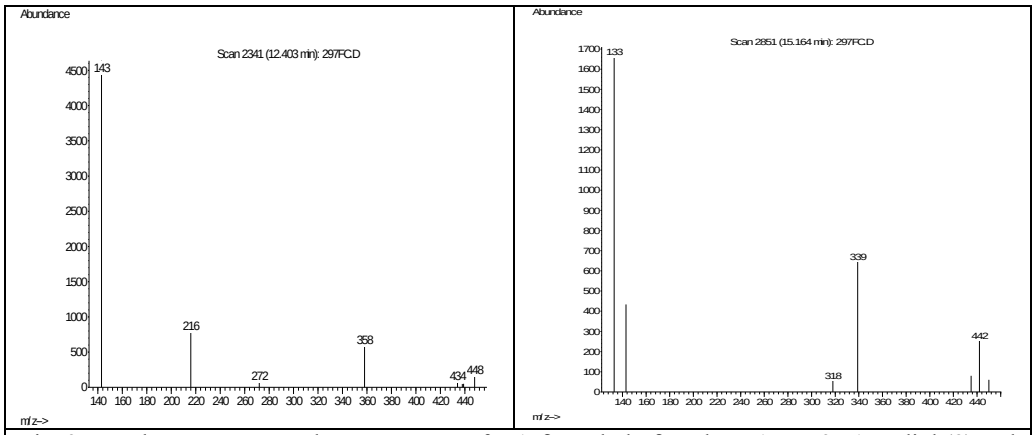


Fig. 3. Ion chromatogram and mass spectrum for 17β-methyl-5β-androst-1-ene-3α,17α-diol (**3**) and 17β-hydroxymethyl,17α-methyl-18-norandrost-1,4,13-trien-3-one (**5**) in the suspicious sample after solid-liquid extraction

6. Conclusions

- Extraction methods and complex analytical techniques were used allowing proving the abuse of Metandienone by confirming the two long-term metabolites.
- The efficient removal of interferences due to biological matrix by solid-liquid extraction followed by removing the free fraction and extracting the metabolites of Metandienone in n-pentane determined a more precise identification of interest compounds.
- The metabolite 17β-methyl-5β-androst-1-ene-3α,17α-diol has been in the focus of the determination of Methandienone abuse by GC/MS approaches due to the long-term retrospective accomplished with it as well as its good gas chromatographic behaviour obtained after derivatization. Now it becomes possible to have good confirmation results for a second long-term metabolite, 17β-hydroxymethyl,17α-methyl-18-norandrost-1,4,13-trien-3-one. The meeting the identification criteria for more long-term metabolites of the same parent compound, could be used for a fair decision in doping control.
- For the confirmation of Metandienone abuse we consider more efficient to apply solid-liquid extraction and gas chromatography coupled with mass spectrometry analysis in current control doping.

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