Romanian Biotechnological Letters *Vol. 14, No. 4, 2009, pp. 4538-4544*
Copyright © 2009 Bucharest University Printed in Romania. All rights reserved
Romanian Society of Biological Sciences

ORIGINAL PAPER

THE RETROSPECTIVE DETECTION OF STANOZOLOL ABUSE IN DOPING CONTROL

Received for publication, April 15, 2009
Accepted, July 15, 2009

VÂJIALĂ ILEANA*, ZORIO MIRELA
National Anti-Doping Agency, Doping Control Laboratory, 37-39 Basarabia Blvd, Sector 2, 022103, Bucharest, Romania
telephone: + 40 726 48 50 29; e-mail: i.vajiala@anad.gov.ro

Abstract

In addition to medical administration Stanozolol is an anabolic androgenic steroid which is often abused in sport to enhance performance. Consequently, the doping control laboratories daily screen for the main urinary metabolites of Stanozolol by advanced detection techniques such as gas chromatography coupled with high resolution mass spectrometry. Upon screening identification of Stanozolol, GC/HRMS confirmation of the suspicious sample is done by reanalysis of the urine specimen, where a specific immunoaffinity purification procedure (IAC) is used to selectively isolate the long term excreted metabolites of Stanozolol. To declare a positive case on Stanozolol abuse the identification criteria specific to doping control must be fulfilled for one or more metabolite of the same parent compound. Applying this strategy to urine samples collected during an excretion study the present paper shows which one and how far out the specific metabolites could be confirmed long time post administration.

Keywords: Excretion study, confirmation, immunoaffinity chromatography, high resolution mass spectrometry

Introduction

Stanozolol metabolism indicates a quick production of mono- and bis-hydroxy-derivatives, excreted mainly in glucuronide form [1,2]. But the level of the urinary excretion is low enough, i.e. 3-5% out of the total administered amount. The most abundant metabolites identified in the human urine are 16β-hydroxystanozolol, 3'-hydroxystanozolol and 4β-hydroxystanozolol (fig. 1).

The published data [3,4] describe Stanozolol excretion up to 3-4 days after cessation and reveal the maximum excretion rate of its monohydroxylated metabolites between 8 and 17 h after oral administration, depending on the applied dose.

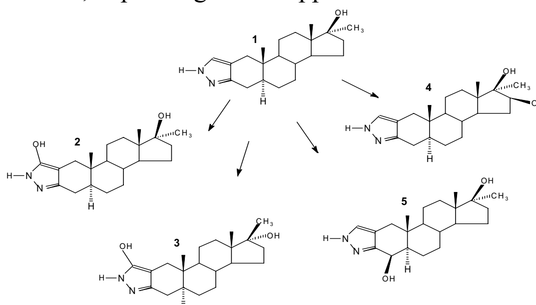

Fig. 1. Stanozolol metabolism (**1**): 3'-hydroxystanozolol (**2**), 3'-hydroxy-17-epistanozolol (**3**), 4β-hydroxystanozolol (**5**) and 16β-hydroxystanozolol (**4**).

It should be mentioned that the data reported within the two excretion studies are based on gas chromatography coupled with low resolution mass spectrometry technique GC/MS (quadrupole), after solid phase extraction (SPE) steps and monitorize as target compounds 3'-hydroxy- and 4β-hydroxystanozolol. The detection by this technique is hampered by the high biological background and does not allow for confirmation of Stanozolol abuse long time post administration. The usual method used by doping control laboratories to prove Stanozolol abuse in sport is based on the detection of its urinary metabolites by GC/(HR)MS, preceded by a specific IAC purification procedure which reduces the biological background and matrix interferences. The use of the HRMS technique increases the sensitivity and specificity of the routine screening analysis and also of the confirmation procedure after specific purification steps [5]. Applying this strategy to urine samples collected during an excretion study the present paper shows which one and how far out the specific metabolites could be confirmed long term post administration with the final aim to demonstrate the retrospective detection of Stanozolol by GC/HRMS.

Material and methods

Materials

The reference substances for Stanozolol metabolites and internal standard 4α-hydroxystanozolol were purchased from National Analytical Reference Laboratory (NARL, Australia). The derivatization agent N-methyl-N-trimethylsilyltrifluoroacetamide MSTFA (for gas chromatography) was supplied by Merck, and the enzyme β-Glucuronidase from *E.coli* was from Roche Diagnostics Manheim. Amberlite XAD_2 resin was purchased from Supelco, USA, and the immunoaffinity gel from Laboratoire d'Hormonologie, Marloie, Belgium. All chemicals and solvents were of analytical grade and were purchased from Sigma and Merck.

Drug administration

As human subjects were involved, the study presented in this paper was approved by the Ethics Commission within National Institute for Sport Research. A therapeutically dose of 40 mg Stanozolol/day was orally administered to a male subject (30 years old, 62 kilos), during a 14 days cure for a bones disease. The subject remains anonymous and his urine samples were collected daily between the 3rd and 43rd day post-application.

There have been also used routine doping control samples that were reported as positive on Stanozolol abuse, following intramuscular injection of 150mg Stanozolol (3 ampoules x 50mg) to a male athlete (22 years old, 60 kg). In compliance with the bioethics and identity confidentiality principles only the urine samples on 46th, 50th and 52nd day post-administration were collected. For stopping the bacterial activity all the samples were added 1g/L NaN_3 and were stored frozen.

Isolation of Stanozolol metabolites for screening analysis

The method developed within the Doping Control Laboratory [6] was adopted after Schänzer and Dönike [5,7]. The extraction procedure for anabolic steroids involves the isolation from 2mL of urine of the steroidic compounds excreted both in conjugated and free form (total fraction). After enzymatic hydrolysis with β-Glucuronidase from *E.coli*, (1h to 50°C), *tert.*-buthylmethyl ether extracted residues were derivatized with reagent mixture MSTFA-NH_4I-ethanethiol (100:0.2:0.6, v/w/v) by heating at 60ºC for 15 min to yield the trimethylsilylated derivatives.

Selective isolation of Stanozolol metabolites for confirmation analysis

The suspicious samples were prepared according to the procedure described by Schänzer & al. [5], together with blank urines and negative urines spiked with pure Stanozolol metabolites solutions as reference samples (concentrations 2-4ng/mL). The urine samples (8-

10mL) were applied on Amberlit XAD_2 resin and the absorbed steroidic compounds were eluted with methanol. After enzymatic hydrolysis with β-Glucuronidase from *E.coli*, (1h to 50°C), the dried ether extracts have been purified by immunoaffinity chromatography (IAC) on glass Econocolumn (Biorad), containing 1mL Sepharose CNBr4B on which the anti-methyltestosterone 3 CMO-BSA antibody was bound. After elution of IAC column, a solution of 4α-hydroxystanozolol 0.1ppm (5ng in 50μL) was added as internal standard in all the samples. The dried methanolic extracts have been derivatized with MSTFA/Imidazole mixture (100:2, v/w) by heating at 60ºC for 15 min.

GC/HRMS analysis

High resolution mass spectrometry experiments were performed with a reverse geometry double focusing mass spectrometer MAT 95XP ThermoFinnigan coupled to an Agilent Technologies 6890N gas chromatograph. On a cross-linked 100% dimethylpolisiloxan capillary column (HP-ULTRA1, J&W Scientific), length 17m, i.d. 0,2mm, film thickness 0,11μm, 1μL of derivatized extracts was injected in pulsed splitless mode. The carrier gas was helium at a constant flow of 1.6 mL/min. Temperatures of injector and transfer line were set at 300^0C. Oven temperature was initially 200^0C, ramped by 15^0C/min to 310^0C and held for 2 min.

The ions were formed by 70eV EI ionization (1mA emission current). The ion source was held at 230^0C. High resolution selected ion monitoring was performed by electric field switching and the continuous calibration of the masses, using a fluorocarbon reference compound (fc_5311). The mass resolution has been adjusted to 5000 and the electrons multiplier was set to 1.8kV. The MS was operated in the multiple ion detection mode (MID) with a single group containing 6 mass fragments characteristic to 3'-hydroxystanozolol and 4β-hydroxystanozolol metabolites, as well as to the internal standard 4α-hydroxystanozolol - m/z 471.3227; 472.3305; 520.3462; 545.3415; 560.3650; 562.3660. The ions were registered with scan cycle time of 0,33sec.

The peaks in the chromatograms presented in this paper are characterized by retention time (RT), height (AH) and signal/noise ratio (SN), parameters selected from Xcalibur software application of MAT 95 XP system. For confirmation, criteria of identification requested by World Anti-Doping Agency [8] were applied, using Excel spreadsheets. The relative ion abundance of at least three diagnostic ions should fulfill the acceptable ranges presented in Table 1.

Table 1. Maximum tolerance windows for relative ion intensities according to WADA documents [8]

Relative abundance (% of base peak)	EI-GC/MS	CI-GS/MS; GC/MS^n; LC/MS; LC/MS^n
>50%	$\pm$10% (absolute)	$\pm$15% (absolute)
25% - 50%	$\pm$20% (relative)	$\pm$25% (relative)
<25%	$\pm$5% (absolute)	$\pm$10% (absolute)

Results and discussions

Oral administration

The monitored metabolites are 3'-hydroxy (fig.1, **2**) and 4β-hydroxystanozolol (fig.1, **5**). All the samples collected post-oral administration were analyzed in routine screening tests [6], where the GC temperature program does not allow for separation of the two metabolites, thus the chromatographic signal is common for both of them. The plot of the abundances of the base peak m/z 545.3415 (100%) against time, starting with the 3rd day post-application (fig.2), shows the dynamics of the excretion of the two co-eluting metabolites. After a

significant decline up to the 6th day, the curve tended to stabilize. Even the intensity of the signals on the plateau zone is very low (the upper right graph in fig.2) all the samples collected might be considered suspicious on Stanozolol abuse upon screening identification. For the decision making process, the samples collected starting with day 6 were re-analyzed by GC/HRMS technique, after IAC purification, in order to prove which one and how far out the specific long-term excreted metabolites could be confirmed.

A faster ramp of 15°C/min applied to the chromatographic oven enables separation of the two metabolites. Figure 3 (*above*) presents the GC/HRMS chromatograms, after IAC purification, of the sample collected in the 6th day post administration. The signals belong to the two monitored long-term excreted metabolites – 3'-hydroxystanozolol (RT 10.44 min) and 4β-hydroxystanozolol (RT 10.52 min), as well as to the internal standard 4α-hydroxystanozolol (RT 10.61 min).

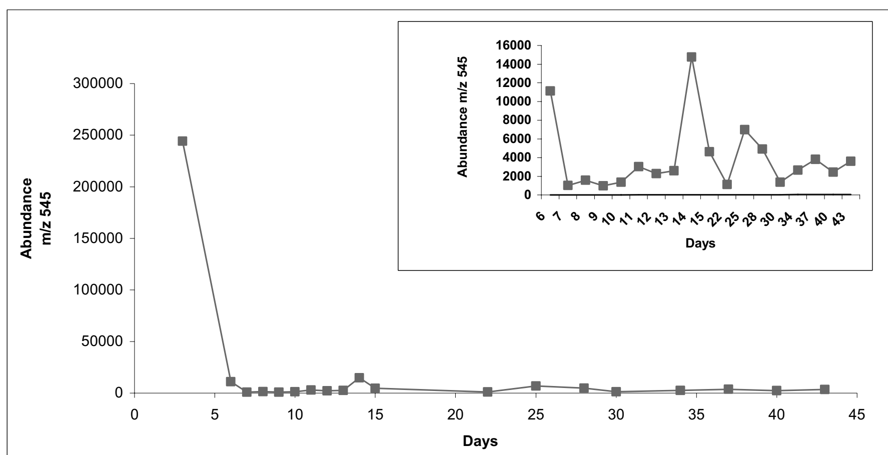

Fig. 2. The plot of the common peak m/z 545.3415 *versus* time, up to 43 days after oral application of 40 mg Stanozolol, by GC/HRMS screening analysis

In order to assess the identification criteria, retention times (RT) and signals heights (AH) of four characteristic ions in chromatograms of reference urine spiked at 4ng/mL (fig. 3 below) and of the suspicious sample (fig. 3 above) are introduced in Excel spreadsheets (Table no.2).

The relative abundances of the signals towards base peak's height, considered 100%, are situated within the acceptance range for all the selected m/z fragments.

The criteria were not met for 4β-hydroxystanozolol metabolite but the sample collected in the 6th day can be declared positive in doping control based on metabolite 3'-hydroxystanozolol compliance with the identification criteria.

In the samples collected in the next days the identification criteria were met for both 3'-hydroxy- and 4β-hydroxystanozolol compounds, with the exception of the sample collected in the 9th day where both metabolites were out of the range.

Beginning with the sample collected in the 13th day, Stanozolol abuse could not be confirmed anymore because of the reduced signals and after the 16th day any Stanozolol metabolites have been detected.

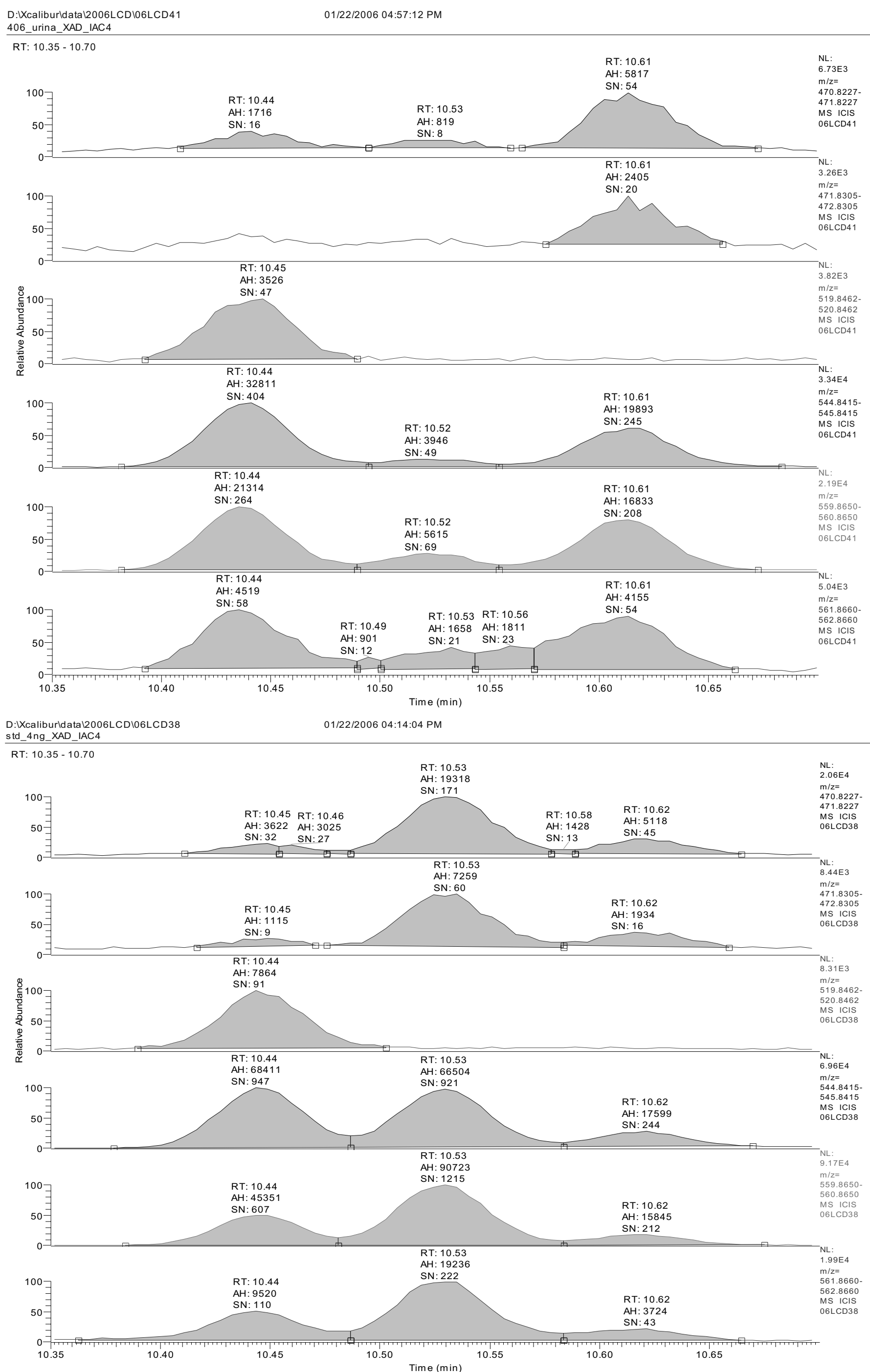

Fig. 3. The chromatogram of day 6 post-oral administration sample (*above*) and of 4ng/mL spiked reference urine (*below*) by GC/HRMS after IAC purification (3'-hydroxystanozolol RT 10.44 min, 4β-hydroxystanozolol RT 10.52 min)

The estimated concentrations of each metabolite in all the analyzed samples are plotted against time in figure 4.

It can be noticed that 3’-hydroxystanozolol is dominant (70-90%).

Table 2. The confirmation of 3’- hydroxystanozolol metabolite in day 6 sample

Reference 3'OH-Stanozolol (4ng/mL)					Day 6 sample_IAC		
TR (min)	m/z	Peak's height	Relative abundance (%)	Accepted range	Relative abundance (%)	Peak's height	TR (min)
10.44	545	68411	100.00		100.00	32811	10.44
	520	7864	11.50	6.5-16.5	10.75	3526	
	560	45351	66.29	56.29-76.29	64.96	21314	
	562	9520	13.92	8.92-18.92	13.77	4519	

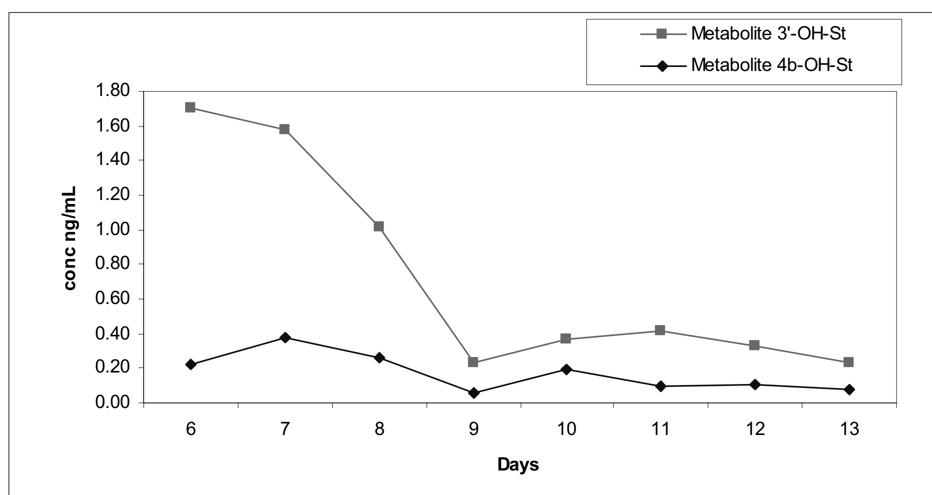

Fig. 4. The plot of metabolites 3’-hydroxystanozolol and 4β-hydroxystanozolol concentrations, between days 6 and 13 after oral application of 40 mg Stanozolol

Intramuscular administration

The three samples collected after IM administration were analyzed following the screening and confirmation procedures already described. At the retention time corresponding to the chromatographic elution of Stanozolol metabolites, signals of reduced intensity were obtained in GC/HRMS screening analysis. After IAC purification step, both long term excreted metabolites were detected in the samples collected in the 46th and 52nd days after IM administration, but the identification criteria were met only for 3’-hydroxystanozolol metabolite. Even present the response of 4β-hydroxystanozolol was out of the range. For the sample collected in the 50th day the identification criteria were not met for any metabolite.

Conclusions

Gas chromatography coupled with high resolution mass spectrometry technique provides an extended detection period depending on the administration way. After oral administration of 40 mg/day for 14 days, good confirmation results were obtained up to the

12th day post-administration, except the 9th day. In the sample collected subsequently (day 13 to 43), although Stanozolol metabolites were detected by GC/HRMS technique the identification criteria specific to doping control were not met.

The abuse of Stanozolol in sport can be proved by the confirmation of one ore more metabolites, therefore, with the exception of 9th day, an athlete could be declared positive on Stanozolol abuse up to 12th day post-oral application.

The negative result in the 9th day post oral administration and in the 50th day after IM administration proves that the individual variations of metabolism could alternate the positive and negative findings and it can not be established rules for the critical days, when the athlete might deceive a doping test.

The detection period depends on the administration way. Compared to 12 days after oral administration, the samples collected post-intramuscular injection could be confirmed on day 52 post-application, which proves the literature data that shows that after intramuscular administration Stanozolol is accumulated on adipose tissue from where is released during physical efforts [9]. In samples collected post IM administration, both long-term excreted metabolites were detected in days 46 and 52, but only 3'-hydroxystanozolol met the identification criteria. Metabolite 3'-hydroxystanozolol is excreted in higher concentration, thus constituting the target compound for long-term detection of Stanozolol abuse in sport, by adequate purification strategies and high resolution mass spectrometry.

References

1. R. MASSE, C. AYOTTE, H. BI, R. DUGAL, *J. Chromatography B*, 497:17 (1989).
2. S. POELMANS, *& al., Anal. Chim. Acta*, **473**:39 (2002).
3. HEA-YOUNG, P. CHOO, OH-SEUNG KWON, JONGSEI PARK, *J. Anal. Toxicology*, 14:09 (1990).
4. W. SCHÄNZER, G. OPFERMANN, M. DÖNIKE**,** *J.Steroid Biochem.*, 36:153 (1990).
5. W. SCHÄNZER, P. DELAHAUT, H. GEYER, M. MACHNIK, S. HORNING, *J.Chromatography B*, 687:93 (1996).
6. G. VÂJIALĂ, M. LAMOR, B. CHIȚIMIA**,** *Recent Advances in Doping Analysis* (9), Sport und Buch Strauβ, Köln, 2001, pp. 233-238.
7. M. DÖNIKE, H. GEYER, A. GOTZMANN, M. KRAFT, F. MANDEL, E. NOLTEERNSTING, G. OPFERMANN, G. SIGMUND, W. SCHÄNZER, J. ZIMMERMANN, *Official Proceedings of the International Athletic Foundation Word Symposium on Doping in Sport***,** *Florenz, 1987*, P. BELLOTI, G. BENZI, A. LJUNGQUIST, eds., International Athletic Foundation, Monte Carlo, 1988, pp. 53-80.
8. *** WADA Technical Document, Identification Criteria for Qualitative Assays Incorporating Chromatography and Mass Spectrometry, TD2003IDCR version 1.2/jan1, 2004, http://www.wada-ama.org/.
9. G. VÂJIALĂ, M. LAMOR, *Doping-Antidoping*, FEST, Bucureşti, 2002, pp. 114-137.