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Improvement in detection of 3'OH-stanozolol in human urine using a new ion trap mass spectrometry system

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Abstract

Stanozolol, a synthetic anabolic androgenic steroid, is often abused in sports to enhance performance. Consequently, the determination of the main metabolite (3'-hydroxystanozolol) in urine has been of particular interest in sport drug testing. A sensitive and specific method by GC-MS^2 (VARIAN 4000) has been developed for the identification in urine of 3'-hydroxystanozolol at trace levels.

Introduction

The use of anabolic androgenic steroids (AAS) in sports has been banned since 1974 by the International Olympic Committee (IOC), National and International Sport Federations and more recently, by the World Anti-Doping Agency (WADA)[1]. The anabolic androgenic steroid stanozolol (17β-hydroxy-17α-methyl-5α-androst-2-eno(3,2-c)-pyrazole), was developed in 1959, and its therapeutic properties such as stimulation of protein biosynthesis and treatment of osteoporosis have been described several fold. However, since its anabolic effects have been recognized, stanozolol has been one of the most frequently misused anabolic agents in sports, which has led to numerous adverse analytical findings in doping controls[2]. Currently, the most reliable, sensitive and specific analytical methods for anabolic steroids screening are GC-MS in single ion monitoring (SIM) mode with electron impact (EI) ionization, GC-MS^n and high resolution mass spectrometry (HRMS). The WADA accredited laboratories mainly focus on the main metabolite, 3'hydroxystanozolol, after enzymatic hydrolysis and TMS derivatisation of the urinary extract. The minimum required performance limit according the WADA technical document for the detection of stanozolol

metabolite, 3'hydroxystanozolol, is 2 ng/mL in urine[3]. In this work was developed a sensitive and specific method for detection of 3'hydroxystanozolol by GC-MS VARIAN 4000.

Materials and Methods

Sample extraction and derivatization: 2 mL of urine was pipetted into a screw-cap glass and the internal standard mixture (17α-methyltestosterone, 2,2,4,4-$^2H_4$-etiocholanolone, 16,16,17-$^2H_3$-testosterone, 16,16,17-$^2H_3$-epitestosterone and 2,2,3,4,4-2H5-androsteron-glucuronide) was added. The pH was adjusted to 7 by adding 0.75 mL of a freshly prepared aqueous solution of 0.8 M sodium phosphate and mixing briefly on a vortex-mixer. To the buffer solution 100 µL of β-glucuronidase from *E. coli* are added and hydrolysis is performed for 1h at 50°C. The buffered solution is alkalinized with 250 µL of 20% potassium carbonate solution (pH 10). *tert*-butylmethylether (4 mL) was added, and the tubes were capped and shaken vigorously for 5min and centrifuged at 2000 rpm for 5 min (g = 536,5). The ethereal phases were transferred to another screw-cap glass and evaporated to dryness under nitrogen at 40°C. The residues were dried in a desiccator for at least 40 min before derivatization. The residues were derivatized with 100µl of MSTFA–NH_4I–2-mercaptoethanol (100:2:6, v/w/v) and heated for 20 min at 60°C. Three microliters of each sample were injected into the GC-MS^n system.

Assay validation: This method was validated regarding specificity, limit of detection, limit of quantification, interday precision (repeatability), trueness and linearity.

Apparatus and chromatographic conditions: A VARIAN 4000 System coupled with a VARIAN 3800 gas chromatograph equipped with a 8400 autosampler and a model 1177 split / splitless injector port was used to carry out all experiments. The software Varian MS Workstation (version 6.6) control all instrumentation, data processing and reporting. Carrier gas was Helium 6.0. VF-1ms capillary column (100% dimethylpolysiloxane, 17m, 0.25mm I.D., film thickness 0.10µm). Injector temperature was 280°C. Injection mode: 3µL split 1/5; septum purge 2mL/min. A splitless 1177 siltek deactivated restek injector liner (6.5 mm x 4 mm ID) and an internal volume of 0,9 mL was used. The GC temperature programming rates were as follows: initial column oven temperature 140°C then increased with a temperature program of 40°C / min, to a temperature of 310°C which was held for 2 min. The transfer line temperature was 300°C, the trap temperature was 230°C and the manifold temperature

was 50°C. The MS instrument was operated in the electron impact ionization mode at 70eV and product ion scan was used as detection mode. For collision induced dissociation in MS^2, helium was used as collision gas.

Results and Discussion

VARIAN 4000 Ion Trap Tandem Mass Spectrometry presented four basic operations: ion formation and matrix ion ejection, precursor ion isolation, product ion formation through collision induced dissociation and product ion mass scanning.

Fragmentation of the precursor ion is performed by collision-induced dissociation (CID) with helium, the carrier gas which fills the ion trap. In the case of the TMS derivative of 3'hydroxystanozolol, the criterion followed for the election of the parent ion were those of selectivity and intensity of the ion, ions with the highest possible m/z were selected. Indeed, the parent ion chosen was not present in the background and did not commonly interfere. CID fragmentation can be performed using non-resonant or resonant excitation mode. Once the excitation mode has been selected, the main parameters determining the fragmentation behavior of an ion are excitation time, dissociation energy and the storage radio frequency.

The best conditions to analyse 3'hydroxystanozolol were optimizated in urine spiked. The instrumental conditions for the detection of 3'hydroxystanozolol (segment three) and the internal standard (methyltestosterone – segment two) are indicated in table 1. In this case, the m/z 545 was chosen as the precursor ion for fragmentation in the trap. By using helium as a collision gas, the fragmentation of the precursor ion was carried out to produce the product ions (m/z 455 and m/z 254).The data demonstrated good performance for all parameters evaluated during the validation assay. The limit of detection, defined as the lowest concentration where a substance can be detected (signal to noise $\geq$ 3) and the limit of quantification was based on a S/N ratio at least five times greater than any interference in blanks at the retention time of the analyte. The results for limit of detection, was 0.05 ng/mL and limit of quantification, was 0.2 ng/mL both criteria were established using the mass trace m/z 387. According to WADA rules, for non-threshold compound, any amount configure an adverse analytical finding (AAF). There are no chromatographic peaks with S/N > 3 interfering with 3'-hydroxystanozolol or internal standard. Regression analysis of the correlation between the chromatographic peak areas ratio of 3'-hydroxystanozolol / internal standard versus known concentrations of analyte yielded a linear correlation over the concentration range analyzed (0.2 – 2.0 ng/mL and 10.0 – 100.0 ng/mL). The corresponding

correlation (r^2) for the curves prepared were 0.992 (0.2 – 2.0 ng/mL) and 0.990 (10.0 – 100.0 ng/mL). Interday precision (repeatability) and trueness are presented in table 2.

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Table 1: GC-MS-MS parameters used for fragmentation.

MS Acquisition Parameters	*Segment one 0-3.2 min*	*Segment two 3.2-4.0 min*	*Segment three 4.0-4.5 min*
Ionization mode	Filament off	EI Full Scan	EI MS/MS
Mass range	-	250-450 m/z	240-555 m/z
Target	-	40,000	10,000
Max. ionization time	-	25,000 µsec	65,000 µsec
Scan time	-	0.15 sec/scan	0.40 sec/scan
EM Voltage offset	-	0V	300V
Filament current	-	30 µAmps	50 µAmps

Table 2: Assay validation results to 3'-OH-stanozolol

Concentration (ng/ml)	CV (%)	Trueness (%)
0.2	4.7	109.1
0.5	13.9	97.3
5.0	5.0	100.5