

Fernandez-Alvarez M<sup>1</sup>, Lu J<sup>2</sup>, Cuervo D<sup>1</sup>, Xu Y<sup>2</sup>, Muñoz-Guerra J<sup>1</sup>, Aguilera R<sup>1</sup>

## Detection of new Oral-Turinabol metabolites by LC-QToF

Laboratorio de Control del Dopaje, Agencia Española de Protección de la Salud en el Deporte (AEPSAD), Madrid, Spain<sup>1</sup>;  
National Anti-Doping Laboratory, China Anti-Doping Agency, Beijing, China<sup>2</sup>

### Abstract

Oral-Turinabol (DHCMT, dehydrochloromethyltestosterone) is a synthetic anabolic androgenic steroid (AAS) prohibited in sports by the World Anti-Doping Agency. In the present work, accurate mass measurements by liquid chromatography coupled to a quadrupole time-of-flight (LC-QToF) system have been used in order to investigate new DHCMT metabolites.

Nine potential biomarkers of Oral-Turinabol consumption, including glucuronide-conjugated and unconjugated compounds, have been detected and tentatively identified after weak basic liquid-liquid extraction (LLE) of post-administration urine samples with no previous hydrolysis step. Chromatographic peaks for such metabolites were found by using the theoretical accurate mass of the corresponding [M-H]<sup>-</sup> ion as target in full scan experiments. Subsequent analyses in targeted MS/MS mode were carried out in order to obtain additional structural information for the compounds of interest. According to the obtained results, hydroxylation of the parent steroid in one or more positions seems to be the most common metabolic pathway. In these preliminary experiments, some of the metabolites could be detected during more than one week after the intake.

According to our bibliographic search, no previous studies regarding the detection of Oral-Turinabol consumption by liquid chromatography-mass spectrometry have been reported. Results obtained from this work will be incorporated in the regular screening method of our laboratory using a liquid chromatography-triple quadrupole (LC-QQQ) system and new detection windows will be established in order to detect DHCMT administration.

### Introduction

Gas chromatography-mass spectrometry (GC-MS) is the common method for the detection of Oral-Turinabol administration. The metabolism of DHCMT was first reported by Schubert and coworkers [1, 2]. In addition to the parent compound, they also identified 6 $\beta$ -hydroxy, 16 $\beta$ -hydroxy, and 6 $\beta$ , 16 $\beta$ -dihydroxy-DHCMT. Dürbeck et al. [3] confirmed 6 $\beta$ -hydroxy and 6 $\beta$ , 16 $\beta$ -dihydroxy-DHCMT metabolites. Other metabolites were tentatively identified as 6 $\beta$ , 12-dihydroxy-DHCMT and 17-epi-DHCMT. 6 $\beta$ -hydroxy-DHCMT is commercially available and it has been the most common marker of Oral-Turinabol consumption during years [4]. However, other compounds which can be detected for longer times after administration (4-chloro-3 $\alpha$ ,6 $\beta$ ,17 $\beta$ -trihydroxy-17 $\alpha$ -methyl-5 $\beta$ -androst-1-en-16-one; 4-chloro-18-nor-17 $\beta$ -hydroxymethyl, 17 $\alpha$ -methyl-androsta-1,4,13-trien-3-one; 4-chloro-17 $\alpha$ -methyl-5 $\beta$ -androst-3 $\alpha$ ,16,17 $\beta$ -triol; 4-chloro-18-nor-17 $\beta$ -hydroxymethyl-17 $\alpha$ -methyl-5 $\beta$ -androsta-1,13-dien-3 $\alpha$ -ol; 4-chloro-18-nor-17 $\beta$ / $\alpha$ -hydroxymethyl-17 $\alpha$ / $\beta$ -methyl-5 $\beta$ -androst-13-en-3 $\alpha$ -ol and 4-chloro-18-nor-17 $\beta$ / $\alpha$ -hydroxymethyl-17 $\alpha$ / $\beta$ -methylandrosta-4,13-dien-3 $\alpha$ -ol) seem to be more useful to detect DHCMT misuse [5-6].

### Experimental

5 mg of Oral-Turinabol were orally administered to a healthy male volunteer (Asian, 45 years old, 70 kg). Urine samples were collected prior and after administration (up to 14 days) and stored at -25°C. 2 mL aliquots were extracted by LLE with 5 mL of tert-butylmethylether (Merck) at pH 9.5 (NaHCO<sub>3</sub>/K<sub>2</sub>CO<sub>3</sub>, Merck and Scharlau, respectively). Extracts were evaporated to dryness under N<sub>2</sub> stream and reconstituted in 200  $\mu$ L of bidistilled water (Millipore):acetonitrile (Merck) (9:1).

An Agilent 1290 Series Rapid Resolution LC system (Agilent Technologies) was employed. Chromatographic separation was performed at 50 °C using a Poroshell 120 EC-C18 (2.1 mm I.D. x 50 mm; 2.7 µm) column. The mobile phase consisted of 5mM NH<sub>4</sub>Ac (Scharlau) in water (A) and acetonitrile (B). Gradient elution program started at 10% B; to 75% B within 12 min; increased to 90% B within 0.1 min; held at 90% B for 1.7 min; finally, returned to initial conditions of 10% B within 0.2 min and re-equilibrated for 3.50 min. Injection volume was 20 µL and flow rate 0.4 mL min<sup>-1</sup>. The detector was an Agilent 6550 iFunnel Q-ToF mass analyser (Agilent Technologies) equipped with a dual AJS ESI source. The instrument was operated in the Extended Dynamic Range (2GHz) mode, which provides a 20k mass resolution. Ionization was performed in positive and negative modes. Nitrogen was used as desolvation and collision gas. Drying gas flow and temperature were set at 12 L min<sup>-1</sup> and 250 °C, respectively, and nebulizer gas pressure at 40 psi. The applied capillary voltage was 4000 V. The fragmentor voltage was set at 150 V. Full scan mass spectral data were acquired from 60 to 1100 m/z at 3 spectra sec<sup>-1</sup>. Reference mass correction was used during the analyses. The instrument operated with the MassHunter Workstation LC/MS Data Acquisition Software version 05.01, and chromatograms were processed with the MassHunter Workstation Qualitative Analysis Software version B.06.00 (both from Agilent Technologies).

## Results and Discussion

Both positive (ESI+) and negative (ESI-) ionization modes have been evaluated. The most interesting results were yielded working with ESI-; therefore, the outcomes included in the present work correspond to those obtained in negative mode. Nine metabolites (see Table 1 and Figure 1) have been tentatively identified on the basis of accurate mass measurements, mass spectrometric information and previous articles regarding DHCMT metabolism [1-6].

Metabolite	Theoretical Accurate Mass (ppm)	RT (min)	Molecular Formula	Metabolic Pathway(s)
M1	509.1948 (1.37)	5.64	C <sub>26</sub> H <sub>35</sub> ClO <sub>8</sub>	Glucuronidation
M2	507.1791 (2.17)	5.02	C <sub>26</sub> H <sub>33</sub> ClO <sub>8</sub>	Wagner-Meerwein rearrangement, Glucuronidation
M3	525.1897 (2.09)	4.54	C <sub>26</sub> H <sub>35</sub> ClO <sub>9</sub>	Hydroxylation, Glucuronidation
M4	523.1740 (2.68)	3.96	C <sub>26</sub> H <sub>33</sub> ClO <sub>9</sub>	Wagner-Meerwein rearrangement, Hydroxylation, Glucuronidation
M5	409.1787* (1.96)	4.25-4.65	C <sub>20</sub> H <sub>27</sub> ClO <sub>3</sub>	Hydroxylation
M6	425.1736* (1.18)	2.38-3.24	C <sub>20</sub> H <sub>27</sub> ClO <sub>4</sub>	Dihydroxylation
M7	423.1580* (1.65)	2.96	C <sub>20</sub> H <sub>25</sub> ClO <sub>4</sub>	Wagner-Meerwein rearrangement, Dihydroxylation
M8	379.1318 (1.58)	1.87	C <sub>20</sub> H <sub>25</sub> ClO <sub>5</sub>	Wagner-Meerwein rearrangement, Trihydroxylation
M9	427.1893* (2.81)	2.50	C <sub>20</sub> H <sub>29</sub> ClO <sub>4</sub>	Reduction, Dihydroxylation

\*Accurate masses are given for the acetate adducts.

Table 1. LC-QToF data obtained for Oral-Turinabol metabolites in full scan experiments and suggestion of the most logical metabolic pathways.

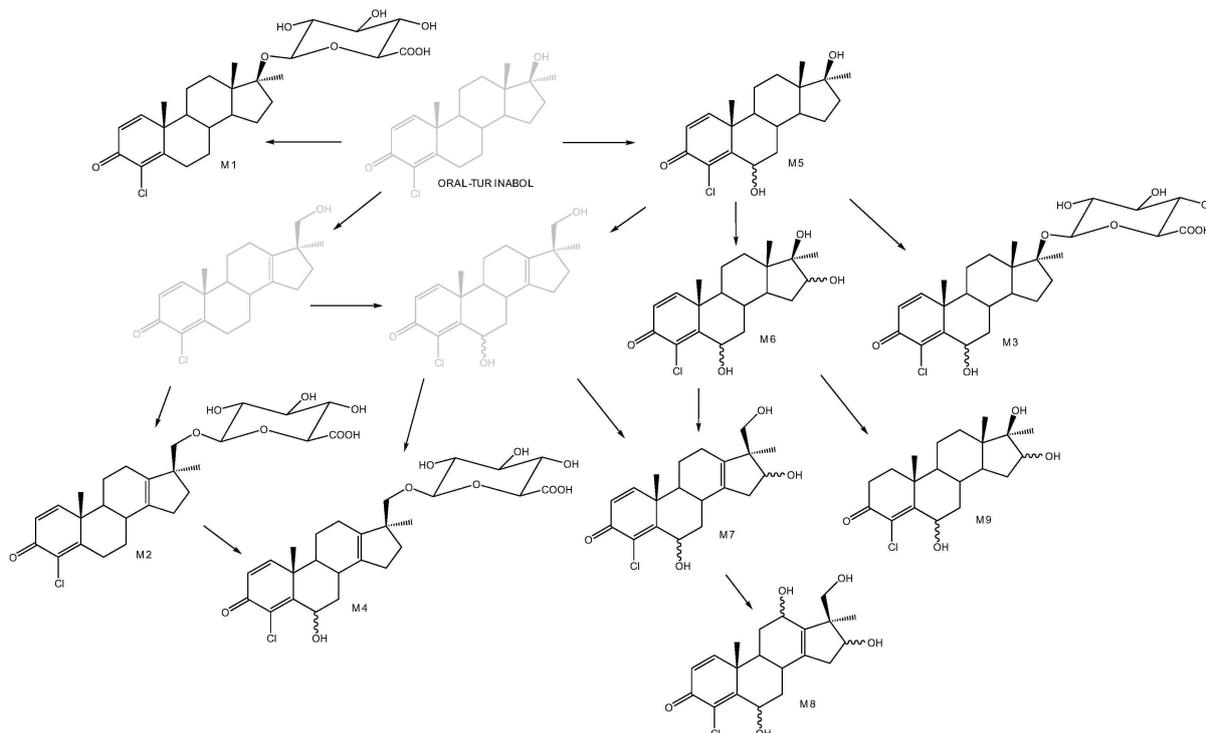


Figure 1. Oral-Turinabol metabolic routes proposed on the basis of accurate mass measurements. Other structures are possible, especially regarding the position of the hydroxyl groups. Several isomers have been detected for M5 and M6, but only one of the possibilities has been plotted.

The accurate mass of Oral-Turinabol M1 is consistent with glucuronidation of the parent compound. Regarding Oral-Turinabol M2, the molecular formula suggests Wagner-Meerwein rearrangement and glucuronidation of DHCMT. Several structures are possible, but a 18-nor-17 $\alpha$ -methyl, 17 $\beta$ -hydroxymethyl-13(14)-ene metabolite is proposed because the corresponding unconjugated compound has been found in previous works [5-6]. Hydroxylation and glucuronidation of Oral-Turinabol are the most logical metabolic pathways leading to metabolite M3. Hydroxylation could take place in position 6 or 16, as reported in previous papers where these hydroxylated metabolites have been detected as free compounds [1-4]. The structure of Oral-Turinabol M4 has been suggested postulating a hydroxylation of M2, which could take place in positions 6 or 16. Figure 2 includes the MS/MS spectra obtained for M1-M4 metabolites.

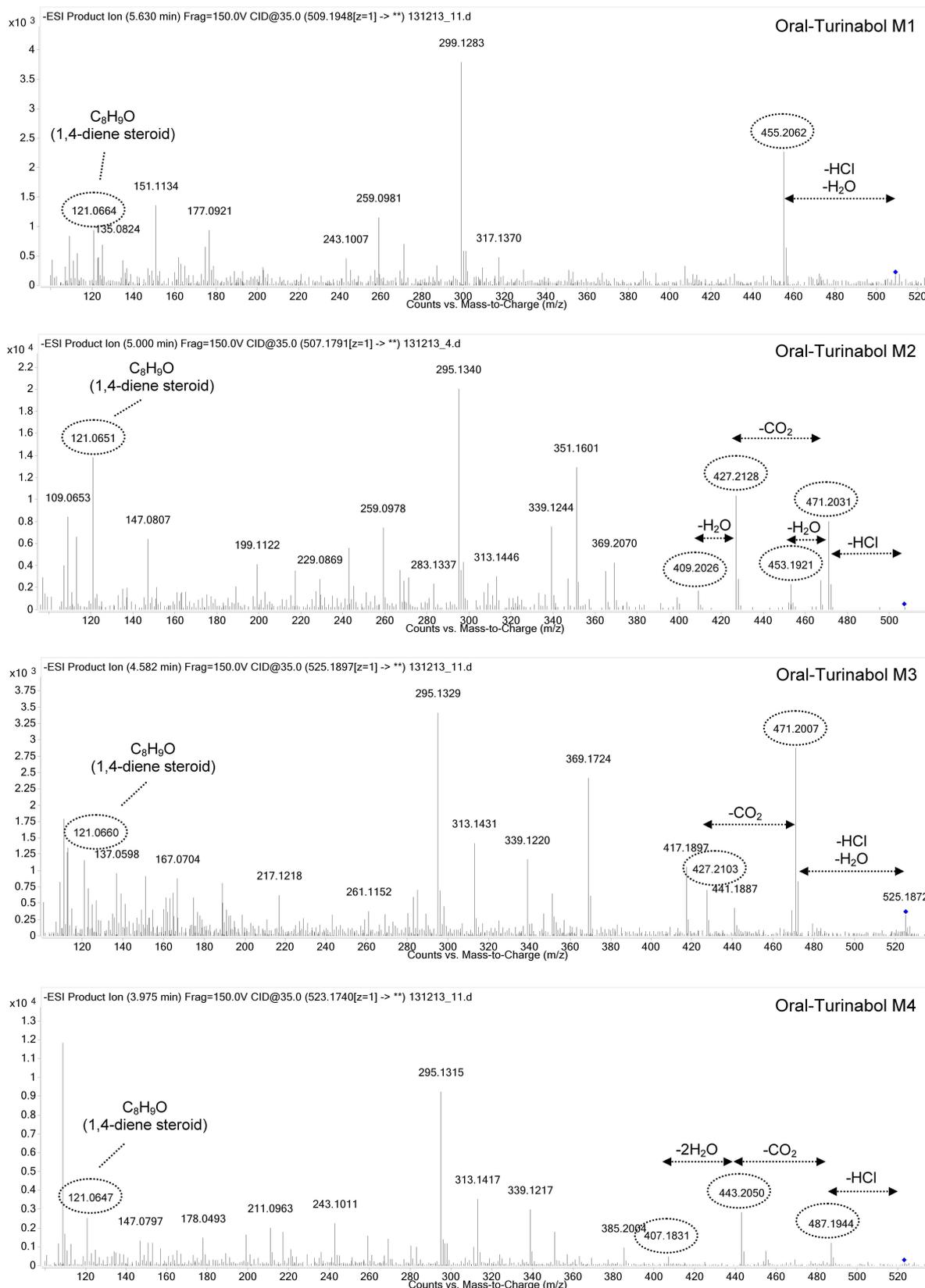


Figure 2: Targeted MS/MS spectra obtained for DHCMT glucuronide-conjugated metabolites (M1-M4).

Hydroxylation of Oral-Turinabol could lead to metabolite M5. Two isomers have been detected: 6OH-Oral-Turinabol (confirmed by injecting the corresponding reference material) and, probably, 16OH-Oral-Turinabol [1-4]. The molecular formula of Oral-Turinabol M6 suggests a dihydroxylation of DHCMT. Three isomers have been detected: 6,16-diOH-Oral-Turinabol and 6,12-diOH-Oral-Turinabol would be the most reasonable structures according to previous studies [1-3]. Since hydroxylation of M2 seems to be a logical metabolic pathway (see M4), we propose M7 as a product of Wagner-Meerwein rearrangement and dihydroxylation of the parent compound. Again, the most susceptible positions for hydroxylation would be 6, 12 and 16. M8 could be a product of M7 hydroxylation. Finally, the structure of M9 has been proposed taking into account that the easiest reducible bond in DHCMT is the 1, 2 one and that 6 and 16 are the most common positions for hydroxylation.

The detection windows obtained for M1-M9 have been plotted in Figure 3.

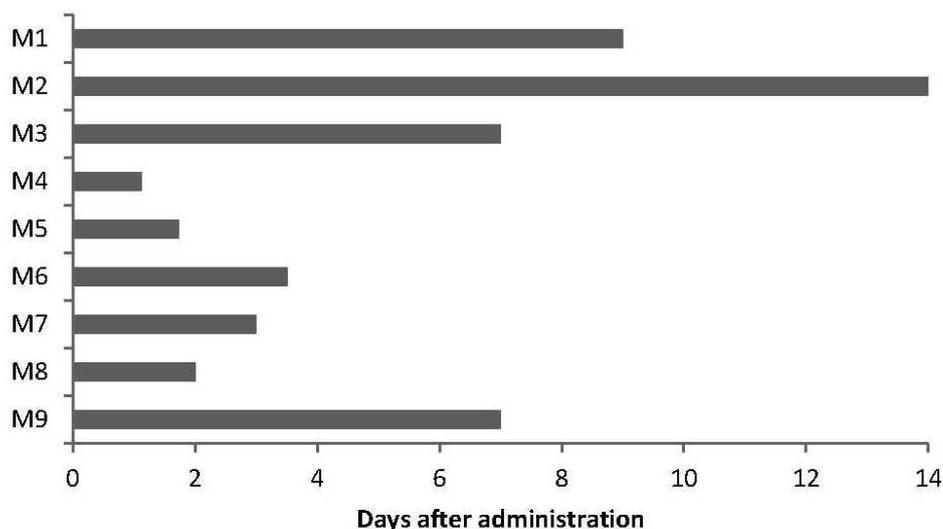


Figure 3: Detection windows of M1-M9 metabolites.

## Conclusions

As far as we know, GC-MS has been the technique employed in all the previous studies regarding Oral-Turinabol metabolism. In this work we have demonstrated the enormous capability of a LC-QToF system for the detection of new DHCMT metabolites. The next step will be the transfer of the obtained results to a LC-QQQ system in order to introduce new metabolites in the scope of our laboratory and improve the detection window of Oral-Turinabol after consumption.

To our knowledge, DHCMT phase II-metabolites are reported here for the first time. Actually, one of these glucuronide-conjugated compounds (Oral-Turinabol M2) seems to be the most interesting metabolite of those identified in this study since it could be detected during two weeks after Oral-Turinabol consumption.

## References

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