

# Detection of Oral-Turinabol Metabolites by LC/MS Q-TOF

## Application Note

Doping Control

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### Abstract

Oral-Turinabol, dehydrochloromethyltestosterone (DHCMT), is a synthetic anabolic androgenic steroid (AAS) prohibited in sports by the World Anti-Doping Agency. We used accurate-mass measurements by liquid chromatography coupled to a quadrupole time-of-flight (LC/MS Q-TOF) system to investigate DHCMT metabolites.

Nine potential biomarkers of Oral-Turinabol consumption, including glucuronide-conjugated and unconjugated compounds, were detected and tentatively identified after weak basic liquid-liquid extraction of post administration urine samples, with no previous hydrolysis step. Chromatographic peaks for the metabolites were found by using the theoretical accurate mass of the corresponding  $[M-H]^-$  ion as the target in full-scan experiments. Subsequent analyses in targeted MS/MS mode obtained more structural information for the compounds of interest. Hydroxylation of the parent steroid in one or more positions seemed to be the most common metabolic pathway. In these preliminary experiments, some of the metabolites were detected more than one week after intake.

According to our bibliographic search, no previous studies on 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 system and new detection windows will be established to detect DHCMT administration.



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## Introduction

Gas chromatography-mass spectrometry (GC/MS) is the common method of choice 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 has been the most common marker of Oral-Turinabol consumption [4]. However, other compounds that can be detected for longer periods after administration seem to be more useful to identify DHCMT misuse [5-6]. These include:

- 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 $\alpha$ -methylandrosta-1,4,13-trien-3-one
- 4-chloro-17 $\alpha$ -methyl-5 $\beta$ -androstan-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
- 4-chloro-18-nor-17  $\beta$ / $\alpha$ -hydroxymethyl-17 $\alpha$ / $\beta$ -methylandrosta-4,13-dien-3 $\alpha$ -ol

## Experimental

Oral-Turinabol (5 mg) was orally administered to a healthy male volunteer (Asian, 45 years, 70 kg). Urine samples were collected before and after administration (up to 14 days) and stored at  $-25^{\circ}\text{C}$ .

Aliquots of 2 mL were extracted by LLE with 5 mL *tert*-butylmethylether (Merck) at pH 9.5 ( $\text{NaHCO}_3/\text{K}_2\text{CO}_3$ , Merck and Scharlau, respectively). Extracts were evaporated to dryness under  $\text{N}_2$  and reconstituted in 200  $\mu\text{L}$  bidistilled water (Millipore):acetonitrile (Merck) (9:1).

An Agilent 1290 Infinity LC was used.

### Chromatographic conditions

Column:	Agilent Poroshell 120 EC-C18, 2.1 $\times$ 50 mm, 2.7 $\mu\text{m}$ (p/n 699775-932)
Mobile phase:	A) 5 mM $\text{NH}_4\text{Ac}$ (Scharlau) in water B) Acetonitrile
Gradient:	10% B, to 75% B in 12 min, to 90% B in 0.1 min, hold for 1.7 min, to 10% B in 0.2 min, re-equilibrated for 3.50 min
Flow rate:	0.4 mL/min
Temperature:	50 $^{\circ}\text{C}$
Injection volume:	20 $\mu\text{L}$

The detector was an Agilent 6550 Accurate-Mass Q-TOF LC/MS with iFunnel technology, equipped with a dual AJS ESI source. Ionization was performed in the negative mode. Nitrogen was used as desolvation and collision gas. Drying gas flow and temperature were set at 12 L/min and 250  $^{\circ}\text{C}$ , respectively, and nebulizer gas pressure at 40 psi. The applied capillary voltage was 4,000 V. The fragmentor voltage was set at 150 V. Full scan mass spectral data were acquired from 60 to 1,100  $m/z$  at 3 spectra/s. Reference mass correction was used during the analyses. The instrument was operated with Agilent MassHunter Workstation LC/MS Data Acquisition Software version 05.01, and chromatograms were processed with MassHunter Workstation Qualitative Analysis Software version B.06.00.

## Results and Discussion

Nine metabolites (see Table 1 and Figure 1) were tentatively identified based on accurate-mass measurements, mass spectrometric information, and previous articles regarding DHCMT metabolism [1-6].

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 an 18-nor-17 $\alpha$ -methyl, 17 $\beta$ -hydroxymethyl-13(14)-ene metabolite is proposed because the corresponding unconjugated compound

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

Metabolite	Theoretical accurate mass (score)	RT (min)	Molecular formula	Metabolic pathway(s)
M1	509.1948 (95.0)	5.64	C <sub>26</sub> H <sub>35</sub> ClO <sub>8</sub>	Glucuronidation
M2	507.1791 (95.3)	5.02	C <sub>26</sub> H <sub>33</sub> ClO <sub>8</sub>	Wagner-Meerwein rearrangement, glucuronidation
M3	525.1897 (94.8)	4.54	C <sub>26</sub> H <sub>35</sub> ClO <sub>9</sub>	Hydroxylation, glucuronidation
M4	523.1740 (83.2)	3.96	C <sub>26</sub> H <sub>33</sub> ClO <sub>9</sub>	Wagner-Meerwein rearrangement, hydroxylation, glucuronidation
M5	409.1787* (97.0)	4.25-4.65	C <sub>20</sub> H <sub>27</sub> ClO <sub>3</sub>	Hydroxylation
M6	425.1736* (93.7)	2.38-3.24	C <sub>20</sub> H <sub>27</sub> ClO <sub>4</sub>	Dihydroxylation
M7	423.1580* (82.9)	2.96	C <sub>20</sub> H <sub>25</sub> ClO <sub>4</sub>	Wagner-Meerwein rearrangement, dihydroxylation
M8	379.1318 (96.2)	1.87	C <sub>20</sub> H <sub>25</sub> ClO <sub>5</sub>	Wagner-Meerwein rearrangement, trihydroxylation
M9	427.1893* (95.6)	2.50	C <sub>20</sub> H <sub>29</sub> ClO <sub>4</sub>	Reduction, dihydroxylation

\*Accurate masses are given for the acetate adducts.

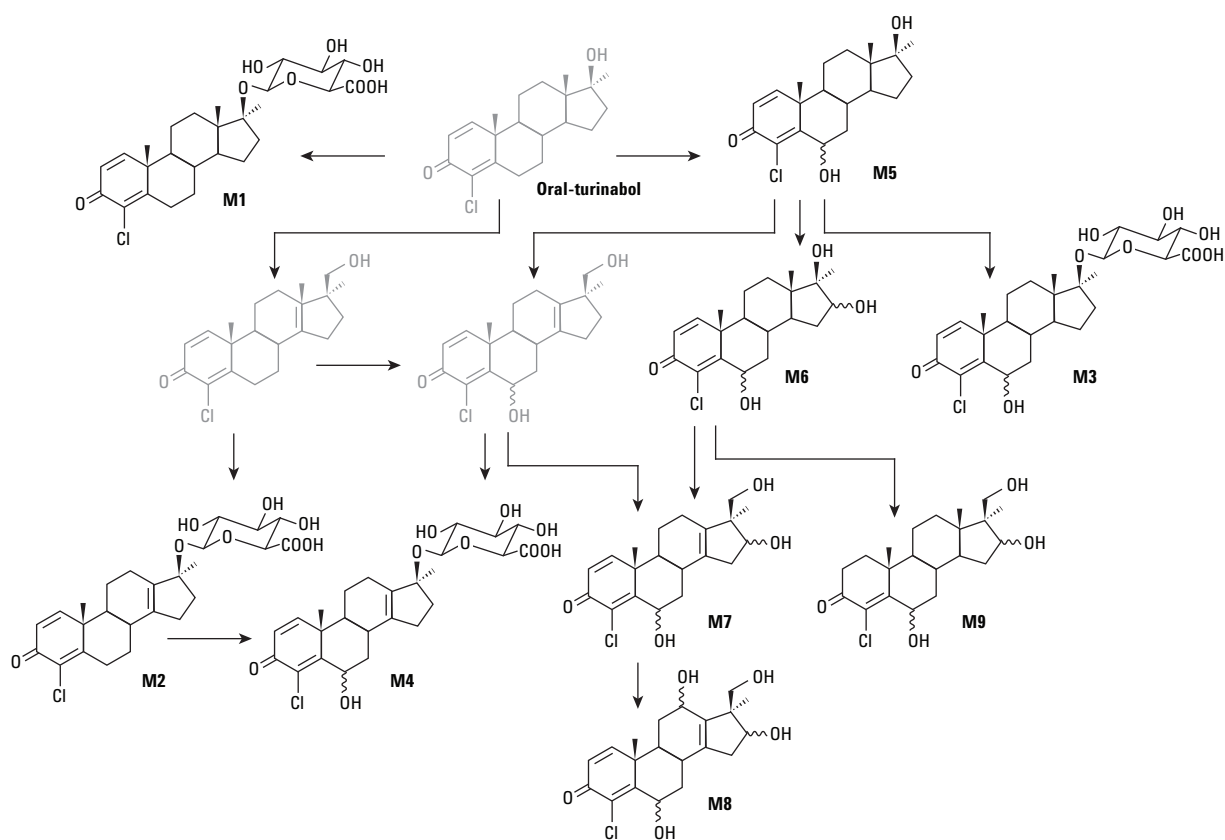


Figure 1. Proposed Oral-Turinabol metabolic routes based on accurate-mass measurements. Other structures are possible, especially regarding the position of the hydroxyl groups. Several isomers were detected for M5 and M6, but only one of the possibilities has been plotted.

has been found in previous work [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.

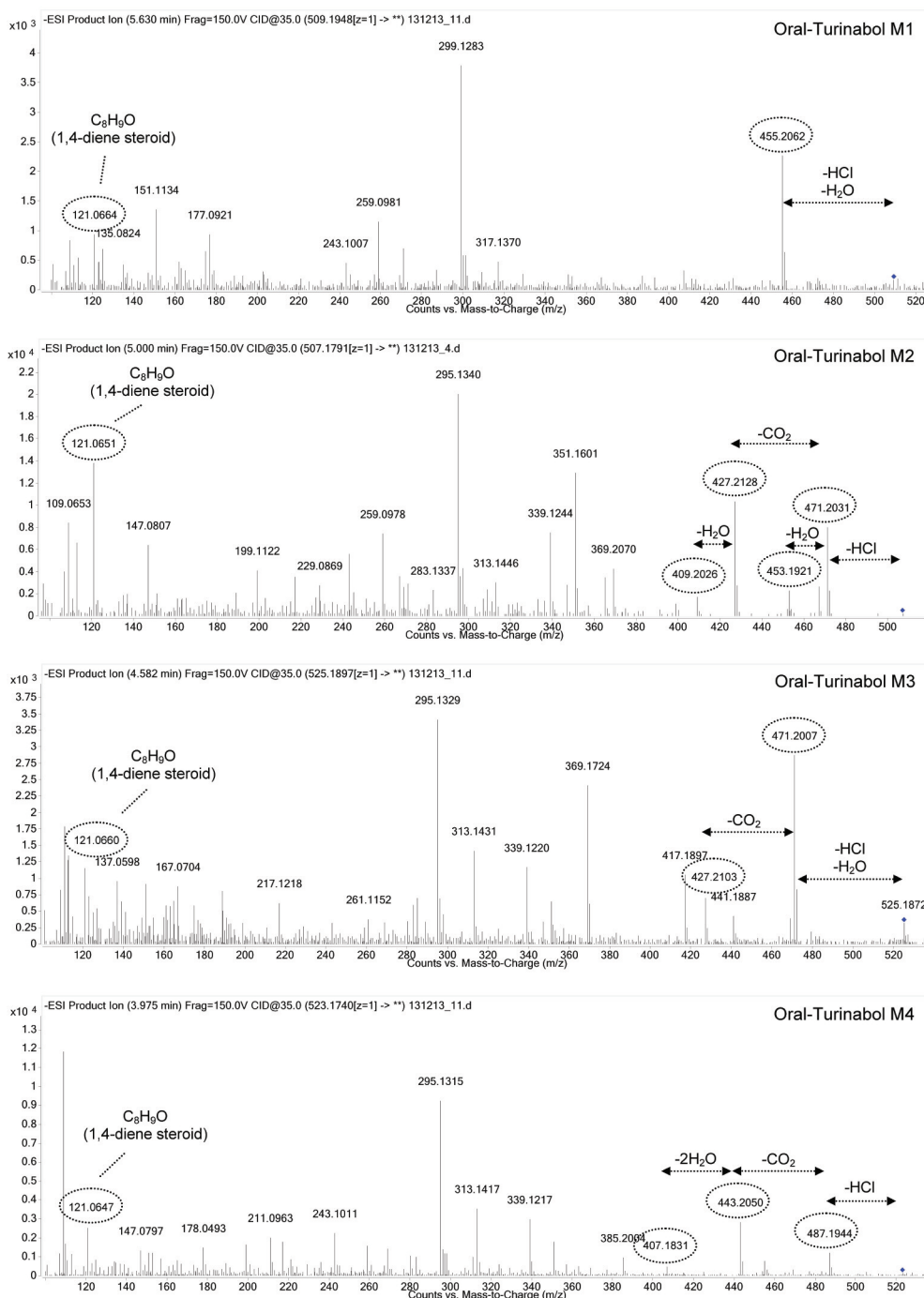


Figure 2. Targeted MS/MS spectra obtained for DHCMT glucuronide-conjugated metabolites (M1-M4).

Hydroxylation of Oral-Turinabol could lead to metabolite M5. Two isomers were 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 were 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 are plotted in Figure 3.

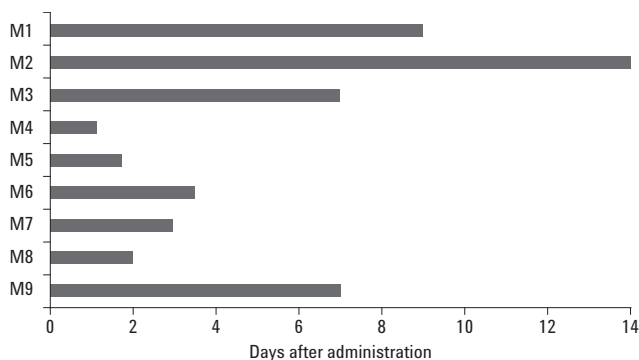


Figure 3. Detection windows of M1-M9 metabolites.

## Conclusions

As far as we can discover, GC/MS was the technique employed in all the previous studies regarding Oral-Turinabol metabolism. In this work, we demonstrate the enormous capability of an LC/MS Q-TOF system for the detection of new DHCMT metabolites. The next step will be to transfer our results to an LC triple quadrupole system to introduce new metabolites in 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. One of these glucuronide-conjugated compounds (Oral-Turinabol M2) seems to be the most interesting metabolite of those identified, since it could be detected at least two weeks after Oral-Turinabol consumption.

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