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Detection of methandienone and stanozolol metabolites: Recent experience at UCLA.

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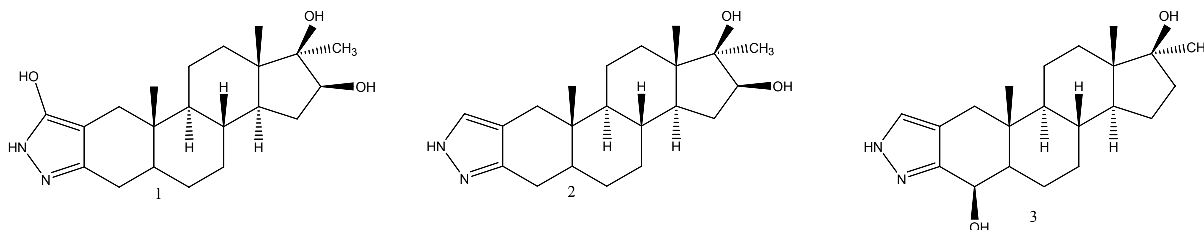
Introduction

Stanozolol (winstrol) and methandienone (methandrostenolone, dianabol) are anabolic androgenic steroids (AAS) on the World Anti-Doping Agency (WADA) prohibited list that are readily available on the black market and through the Internet. According to the WADA 2009 statistics, stanozolol and methandienone are among the most widely abused AAS. Stanozolol was the most abused exogenous AAS and accounted for 208 reported cases (6.3% of all reported exogenous AAS) whereas methandienone accounted for 110 cases (3.3% of all reported exogenous AAS) and ranked third. In the past two years (2009 & 2010) the UCLA Olympic Analytical Laboratory reported 84 stanozolol and 61 methandienone adverse analytical findings. In this study we present the distribution of metabolites for stanozolol and methandienone, and the concentration of stanozolol metabolites for 145 positive cases.

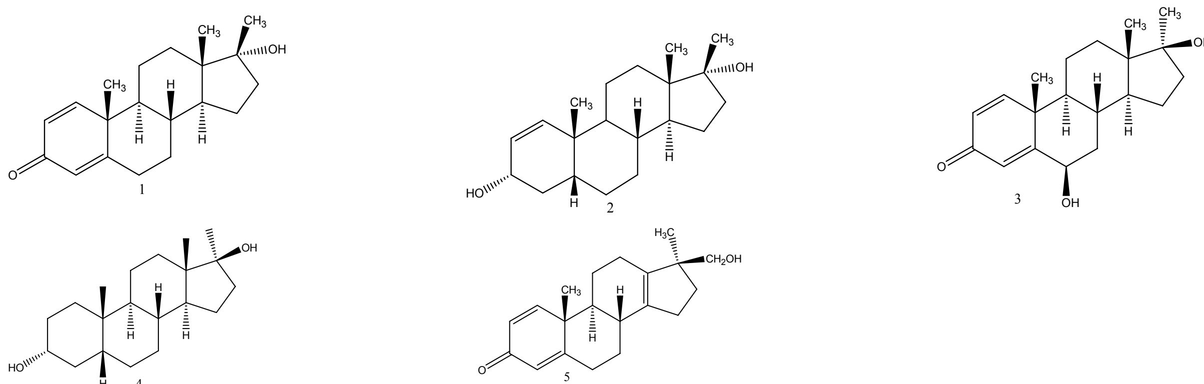
Materials and Methods

<u>Stanozolol and methandienone metabolites</u>. The stanozolol metabolites 3'-hydroxystanozolol (1) and 16β-hydroxystanozolol (2) were monitored in the screening method by LC-MS/MS. 4β-hydroxystanozolol (3) was also monitored during confirmation testing (below).



The methandienone metabolites 17-epimethandienone (1), epimetendiol (2), 6β-hydroxy-methandienone (3), 17α-methyl-5β-androstane-3α,17β-diol (4), and 17β-hydroxymethyl-17α-

methyl-18-norandrosta-1,4,13-trien-3-one (5) were monitored during screening and confirmation by GC-MS (below).



<u>*LC-MS/MS method for stanozolol*</u>. Deuterated internal standard, d3'-hydroxystanozolol, was added to 2.5 mL of urine. Enzymatic hydrolysis was performed using *E coli* beta-glucoronidase in 0.2 M phosphate buffer for 1 hour at 50ºC, followed by liquid-liquid extraction with pentane/ethyl ether at pH 9.5. The organic extract was dried and reconstituted in 50µL of 5 mM ammonium acetate:acetonitrile (65:35). The reconstituted extracts were analyzed by a AB 3000 LC/MS/MS with a Shimadzu 10AD VP dual pump system using a Phenomenex Synergy 5 µm MAX-RP 50 x 2 mm column. The turbo spray ion source was operated in positive polarity mode (Table 1). The limit of detection was 1 ng/mL for all metabolites.

Table 1. Ionization parameters

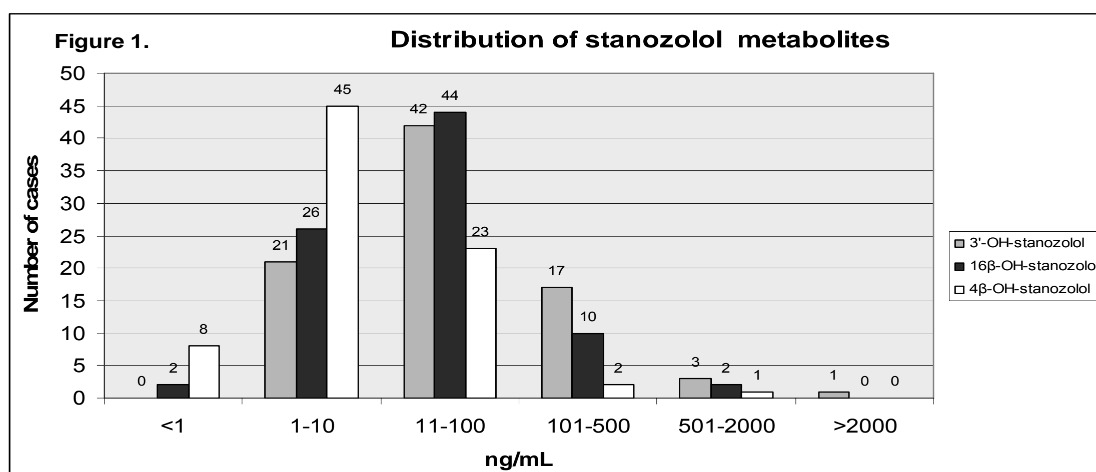
Compound	MRM	Declustering Potential	Collision Energy
d3-3'-OH-stanozolol	348→97	91	62
3'-OH-stanozolol	345→97	46	67
4β-OH-stanozolol	345→145	71	23
16β-OH-stanozolol	345→81	61	77

<u>*GC-MS method for methandienone*</u>. Deuterated internal standard, d3-testosterone was added to 2.5 mL of urine. Enzymatic hydrolysis was performed using E coli beta-glucoronidase in 0.2 M phosphate buffer for 1 hour at 50ºC for 1 hour. The hydrolyzed urine was then applied to an Empore SPE disc cartridge, eluted with ethyl acetate, dried and derivatized with 50 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide/ammonium iodide/dithioerythritol (1000:2.5:5, v/w/w). Derivatized extracts were injected into an Agilent 5973 or 5975, ULTRA 1 column (17 meter, 0.2 mm ID, 0.11 µm film thickness). Injection was done with a

split ratio of 1:10 in selected ion monitoring mode. The limit of detection was 2 ng/mL for 17α-methyl-5β-androstane-3α,17β-diol and 10 ng/mL for the other metabolites.

Results

A total of 62,762 LC-MS/MS screens were performed during the two year period. Of these, 84 urine samples (0.13% of all tested samples) were positive for stanozolol metabolites. In the majority of positive cases (94%) all three stanozolol metabolites were detected during confirmation. In the remaining 6%, only 3'-OH-stanozolol and 16β-OH-stanozolol were detected. 3'-OH-stanozolol was the most abundant metabolite. The range of concentrations for metabolites ranged from less than 1 ng/mL to greater than 2000 ng/mL (Figure 1).



A total of 66,750 GC-MS steroid screens were performed during the same period. Of these, 61 samples (0.1% of all tested samples) were found positive for methandienone metabolites. All five metabolites were detected in 36 of the cases (61%). The frequency of each metabolite is shown in Table 2.

Table 2. Distribution of methandienone metabolites.

Metabolite	Number of Cases Detected	Percentage of Cases detected
17β-hydroxymethyl-17a-methyl-18-norandrosta-1,4,13-trien-3-one (5)	57	97%*
Epimetendiol (2)	58	95%
17-epimethandienone (1)	54	89%
17α-methyl-5β-androstane-3α,17β-diol (4)	53	87%
6β -hydroxymethandienone (3)	51	84%

*Metabolite was monitored in only 59 cases

Due to the limited availability of reference material, metabolite (5) was only quantitated in some of the positive cases. The distribution of metabolites in 8 positive cases where all metabolites were quantitated is shown in Figure 2. Metabolite (5) was in the highest concentration in 7 of 8 cases and was the only detectable metabolite in one case. In one case the most abundant metabolite was metabolite (3). When present, metabolite (4) represented 10% or less of the total methandienone metabolites.

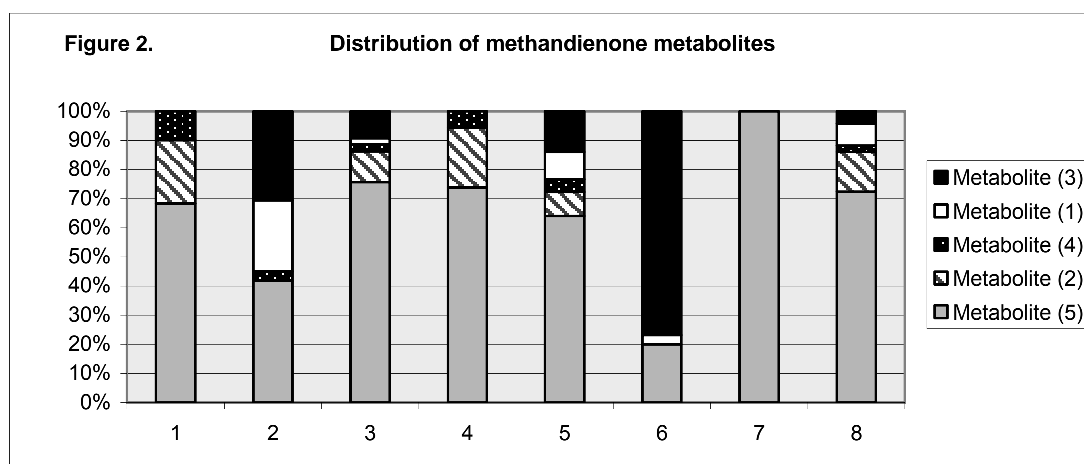


Figure 2. **Distribution of methandienone metabolites**

Summary

Screening for 3'-OH-stanozolol and 16β-OH-stanozolol is sufficient for detecting stanozolol use. For methandienone, none of the metabolites were detected in all the positive cases, indicating that it is prudent to monitor all 5 metabolites for detecting methandienone use.

Acknowledgement

d3-3'-OH-stanozolol and 17β-hydroxymethyl-17a-methyl-18-norandrosta-1,4,13-trien-3-one reference standards were provided by the Institute of Biochemistry, German Sport University, Cologne, Germany.

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