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Effects of ketoconazole on the excretion kinetics of methandienone. An *in vivo* study.

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Abstract

This research is part of a wider project designed to provide more information on the implications that metabolic (phase I and phase II) drug-drug interactions might have on the effectiveness of doping control strategies, currently adopted by the antidoping laboratories, to detect drug intake. In the first part of this project we showed that the *in vitro* metabolic profile of several prohibited substances, e.g. methandienone, was extensively altered in the presence of the azole-antifungal agents ketoconazole, itraconazole, miconazole and of the antidepressant nefazodone. To confirm the occurrence of these observations, we report here the results of excretion studies (methandienone single dose of 5 mg *per os*) with and without the application of ketoconazole (single dose of 200 mg *per os*). The results obtained showed that the urinary levels of methandienone and of most of its metabolites were extensively altered by ketoconazole administration. Furthermore, our data also showed that ketokonazole significantly modified the excretion kinetics and windows of detection of methandienone and most of its metabolites. Notably, also the "diagnostic markers" selected as long-term detected methandienone metabolites were included in this group of effected compounds.

Introduction

Methandienone is one of the most frequently androgenic anabolic steroid detected in sports by WADA accredited anti-doping laboratories as indicated in the WADA statistical reports [1]. Its metabolism was extensively investigated via *in vivo* and *in vitro* studies [2-9]. The metabolites selected as “diagnostic markers” to detect its use are: 6β-hydroxy-methandienone, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5β-androstane-3α,17β-diol, epimethendiol and 18-norepimethendiol, from which the last two allow a long-term detection of methandienone intake. Recently other two metabolites, 20α and 20β-hydroxy-18-normethandienone were identified by Schänzer *et al.* as complementary markers for long-term detection of methandienone use [7-9]. The dehydrogenation, hydrogenation and hydroxylation are catalyzed by the cytochrome P450 (CYP450) enzymatic system, whereas the 17-epimerization as well as the formation of 18-nor-17,17-dimethyl analogs result from the Wagner-Meerwein rearrangement or degradation and rearrangement of 17β-sulphate conjugates. In a previous study we showed that the *in vitro* metabolic profile of several prohibited substances, including methandienone, was significantly altered by the non-prohibited drugs [9]. Although in literature many clinical studies are reported on the capacity of the azole-antifungal agents in altering the activity of the CYP450 enzymatic system, to the best of our knowledge no data are reported in literature on the occurrence and on the potential consequences of these interactions on the analytical strategies followed by the antidoping laboratories to reveal the intake of banned substances. Here the metabolic profile of methandienone was investigated before and after application of ketoconazole.

Experimental

Chemicals and reagents

6β-hydroxy-methandienone, 17-epimethandienone, epimethendiol, 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one, 17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol were from NMI (National Measurement Institute, Pymble, Australia). The 17α-methyl-5β-androst-1-en-17β-ol-3-one and methandienone were purchased from Steraloids (Milano, Italy). The 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one was a gift from the Institut für Biochemie of the Deutsche

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Sporthochschule in Cologne, Germany. The 17α-methyltestosterone (used as internal standard) and ketoconazole were supplied by Sigma-Aldrich (Milano, Italy). All reagents were provided by Carlo Erba (Milano, Italy). The ultrapure water used was of Milli-Q-grade (Millipore, Milano, Italy).
Urine samples from a healthy male subject were collected before and after administration of a single dose of methandienone (5 mg of methandienone *per os*) and after the combined administration of a single dose of methandienone (5 mg of methandienone *per os*) and of ketoconazole (200 mg of ketoconazole *per os*).
Analytical procedure
The pre-treatment of the urine samples consists of an enzymatic hydrolysis (50 µL of β-glucuronidase from *E. coli,* incubation for 1 hour at 55 ºC) and of a liquid/liquid extraction with *tert*-butylmethyl ether at pH 7.4. The organic layer was separated and evaporated to dryness. The residue was reconstituted in 50 µL of mobile phase and 10 µL were injected into the liquid chromatographic-mass spectrometric (LC-MS) systems.
All LC experiments were performed using an Agilent 1200 Rapid Resolution Series HPLC pump with binary gradient system and automatic injector (Agilent Technologies S.p.A, Milano, Italy). Chromatography separation was performed using a Discovery C18 column from Supelco (2.1 x 150 mm, 5 µm). The solvents used were: 0.1% formic acid (A) and acetonitrile (B). The flow rate was set at 300 µL/min. The gradient program started at 10% B and increased to 60% B in 7 min and then, after 6 min, to 100% B in 1 min. The column was flushed for 1 min at 100% B and finally re-equilibrated at 15% B for four minutes. All LC-ESI-MS/MS experiments were performed using an API4000 triple-quadrupole instrument (Applera Italia, Monza, Italy) with positive electrospray ionization. Instrumental parameters were optimized by infusing the standards of methandienone and of the metabolites available as reference material dissolved in mobile phase at a concentration of 10 µg/mL in positive ionization mode. The ion source temperature was set at 500 °C, the curtain gas pressure at 25 psi, the ion source gas 1 pressure at 35 psi, and the ion source gas 2 pressure at 40 psi. The declustering voltage was set at 60 V and the needle voltage at 5500 V.
High-resolution/high-accuracy measurements were performed on an Agilent Technologies 6210 orthogonal acceleration time-of-flight mass spectrometer, equipped with an electrospray ionization source operated in positive ion mode. Nitrogen was used as the drying and nebulising gas. The drying gas flow rate and temperature were 10 L/min and 350°C, respectively. The applied capillary and fragmentor voltages were set at 4000 V and at 150 V respectively. Mass spectra data were collected from m/z 100 to 1100 at a resolution of 20000. Purine (*m/z* 121.0509) and an Agilent proprietary compound (HP0921, *m/z* 922.0098) were used as internal calibrants.

Results and Discussion

Metabolic profile of methandienone
The analysis of the excretion study samples collected after oral administration of a single dose of methandienone (5 mg) using a time-of flight system in full scan mode, resulted in detectable urinary concentration of parent methandienone, epimethandienone, 6β-hydroxymethandienone, epimethendiol and of 8 analytes for which there are no reference standards available in our laboratory and not present in the urine sample collected before drug administration (see Figures 1A-B). The 17α-methyl-5β-androsta-3α,17β-diol, and 17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol were not detected due to poor ionization efficiency in electrospray . The chemical structures of the eight analytes were elucidated analyzing the excretion study samples and the standard solution of methandienone and of the metabolites available as references materials by time-of flight system in product ion scan as acquisition mode at low (20 eV) and high (45 eV) collision energy. The structure specific ions found in the product ion spectra of the known and unknown analytes together with their elemental composition, chromatographic retention times and data reported in literature [2-11] allow to postulate the chemical structures of the unknown compounds (see Table 1).
The excretion profiles in the free and glucuronide-conjugated fractions and the windows of detection of methandienone and its metabolite were obtained analyzing the excretion study samples by triple quadrupole in selected reaction monitoring (SRM) as acquisition mode, set up utilizing the information obtained by the product ion spectra. Most of methandienone metabolites were excreted mainly in the free fraction. Methandienone itself and epimethendiol were exceptions, in fact methandienone was mainly detected in the conjugated fraction and epimethendiol was excreted only as conjugated. Methandienone and most of its metabolites were detectable in urine for more than 24 h with a maximum of excretion generally after 4-24 h from drug administration confirming the data reported by Parr *et al.* [9]. More specifically, methandienone, M10 and 18-normethandienone were detected for four days, epimethandienone, 6-ene-methandienone and 6β-hydroxy-methandienone for five days, the other hydroxylated metabolites for three days, epimethendiol for seven days and the hydroxymethylated metabolites for more than two weeks confirming the data reported in previous studies [7-9].

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Compound	Elemental composition	MW (Da) (calc)	MW (Da) (exp)	Error (Δppm)	SRM (*m/z*)	CE (eV)
Dihydroxy-methandienone (M1, Di-OH-MD))	$C_{20}H_{28}O_4$	332.1988	332.1985	0.9	333/121	35
6β-hydroxy-methandienone (M2, 6β-OH-MD)	$C_{20}H_{28}O_3$	316.2038	316.2035	0.9	317/147; 317/121	30, 35
Monohydroxy- methandienone (M3, OH-MD)	$C_{20}H_{28}O_3$	316.2038	316.2044	1.9	317/121	35
6-ene-methandienone (M4)	$C_{20}H_{26}O_2$	298.1933	298.1930	1.0	299/147; 299/121	35
Methandienone (MD)	$C_{20}H_{28}O_2$	300.2089	300.2083	2.0	301/149; 301/121	30; 35
M5	$C_{20}H_{26}O_2$	298.1933	298.1928	1.6	299/147; 299/121	35
20β-hydroxy-18-normethandienone (M6, 20β-OH-norMD)	$C_{20}H_{26}O_2$	298.1933	298.1936	1.0	299/147; 299/121	30; 35
M7	$C_{20}H_{26}O_2$	298.1933	298.1930	1.0	299/147; 299/121	30; 35
Epimethandienone (M8, EpiMD)	$C_{20}H_{28}O_2$	300.2089	300.2088	0.3	301/149; 301/121	30; 35
Epimethendiol (M9, EMD)	$C_{20}H_{31}O_2$	303.2324	303.2320	1.3	269/121; 269/147	30; 35
M10	$C_{20}H_{26}O$	282.1984	282.1990	2.0	283/121; 283/147	30; 35
18-nor-methandienone (M11, 18-norMD)	$C_{20}H_{26}O$	282.1984	282.1990	2.0	283/121; 283/147	30; 35

Table 1: Elemental composition, molecular weight, characteristic ion transition (SRM), collision energy (CE)

Metabolic profile of methandienone after combined administration with ketoconazole

The urine samples collected after the combined administration of a single dose of methandienone (5 mg) and of a single dose of ketoconazole (200 mg) were analyzed using the SRM method set up in this study. In Figures 1A-C were reported the results obtained analyzing urine samples collected before methandienone administration (Figure 1A), after 24 h from methandienone administration (Figure 1B) and after 24 h from the combined administration of methandienone and ketoconazole (Figure 1C). The urinary levels of methandienone and of most of its metabolites were significantly reduced after ketoconazole administration confirming the *in vitro* data obtained in a previous study [12]. In addition the dihydroxylated metabolite (**M1**) was not detected in the urine samples collected after the combined administration of methandienone and ketoconazole.

Figures 2-3 report the excretion profile and the windows of detection of methandienone and its metabolites after administration of methandienone (solid lines) and after combined administration of methandienone and ketoconazole (dotted lines). The levels of methandienone and of the dehydrogenated and of the hydroxylated metabolites decrease significantly whereas the windows of detection increase, due to a slower kinetics of formation and excretion. On the contrary the kinetics of excretion of the demethylated metabolites is faster and the windows of detection decrease while the urinary levels increase. These observations can be explained considering that ketoconazole is a competitive CYP450 inhibitor, as well as of the uridine diphosphoglucuronosyltransferases, consequently the biotransformation pathways catalyzed by these enzymatic systems (glucuronidation, hydroxylation and dehydrogenation) were temporarily arrested, whereas the metabolic reactions (epimerization and demethylation) in which other enzymatic system (Wagner–Meerwein rearrangement or degradation and rearrangement of the 17β-sulphate conjugates) are involved were induced.

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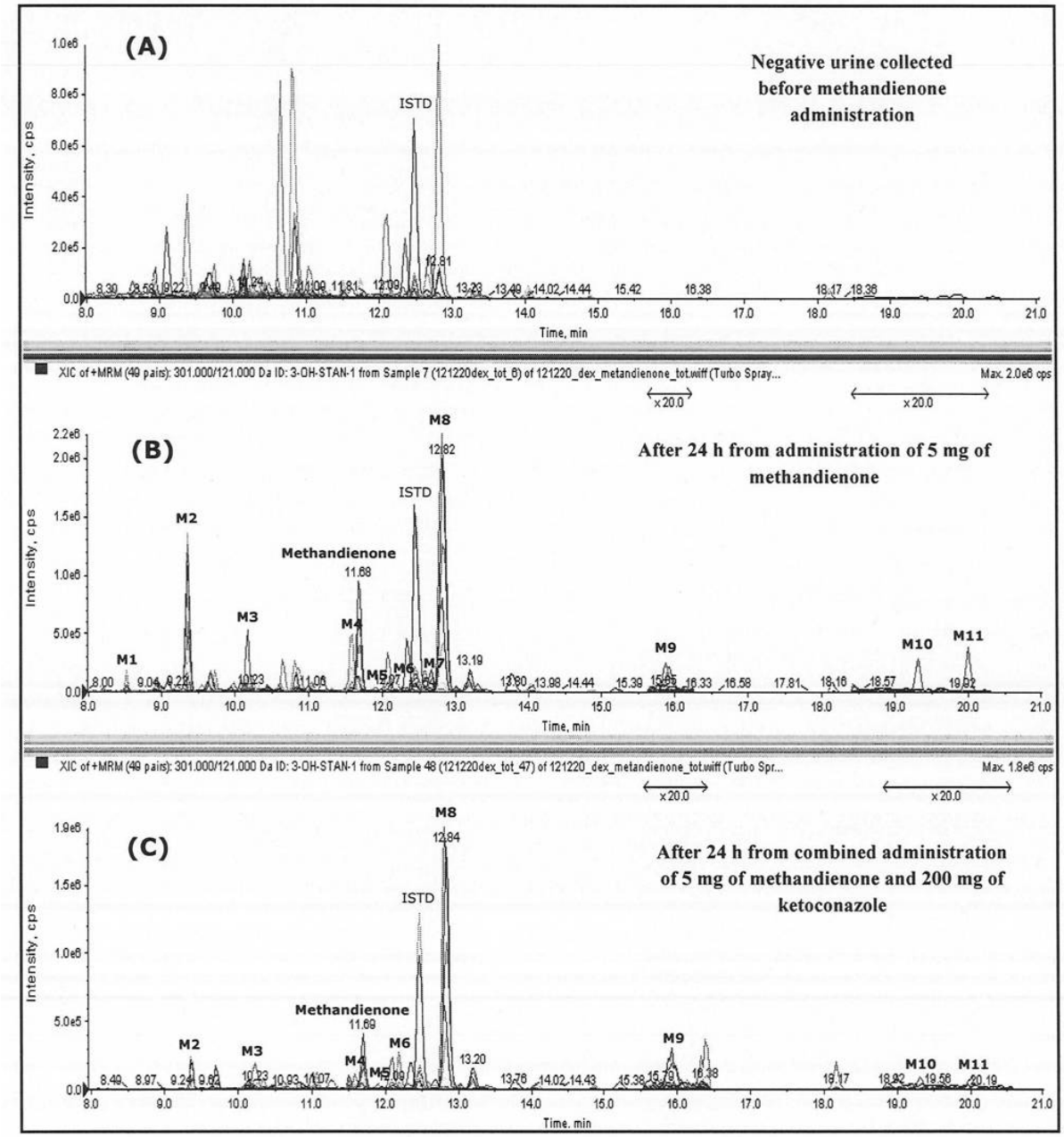

Figure 1: Extract chromatograms of a urine sample collected before the administration of metandienone (negative urine) (A) after 24 h from the oral administration of 5 mg of methandienone (B) and after 24 h from the combined administration of 5 mg of methandienone and 200 mg of ketoconazole (C). Peak identification: M1. Dihydroxy-methandienone, M2. 6β-hydroxymethandienone, M3. Hydroxy-methandienone, M4. 6-ene-methandienone, M5. 20α-hydroxy-18-normethandienone, M6 20β-hydroxy-18-normethandienone, M7. 6-ene-epimethandienone, M8. epimethandienone M9. epimethendiol, M10., M11. 18-normethandienone.

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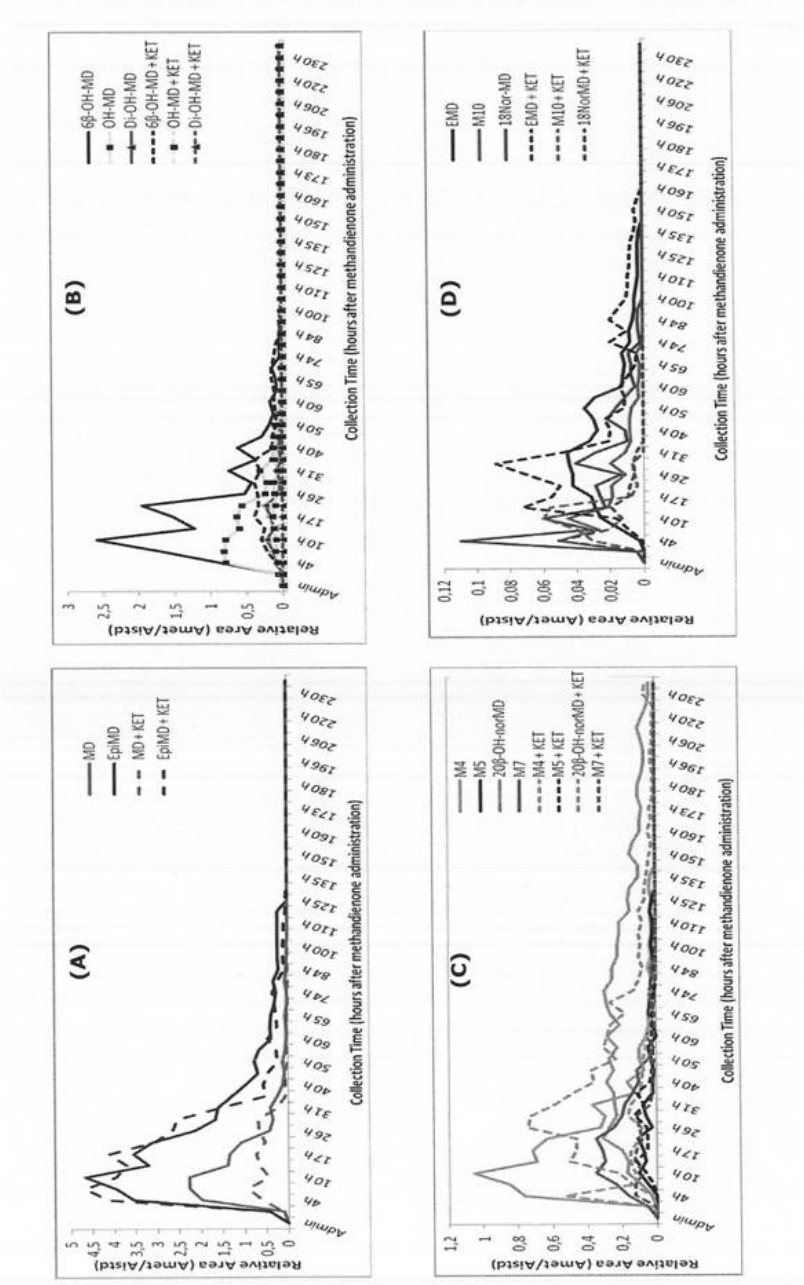

Figure 2: Urinary excretion profile of methandienone and its metabolites after oral administration of 5 mg of methandienone (solid lines) and after combined administration of 5 mg of methandienone and 200 mg of ketoconazole (dotted lines). (A) reports the excretion profiles of methandienone (MD) and epimethandienone (EpiMD); (B) reports the excretion profiles of the mono (6β-OH-MD and OH-MD) and di-hydroxylated metabolites (Di-OH-MD) (6); (C) reports the excretion profiles of the dehydrogenated and hydroxymethylated metabolites (M4, M5, 20β-OH-norMD and M7); (D) reports the excretion profiles of epimethendiol (EMD), and of the demethylated metabolites M10(M10) and 18-nor-methandienone (18NorMD).

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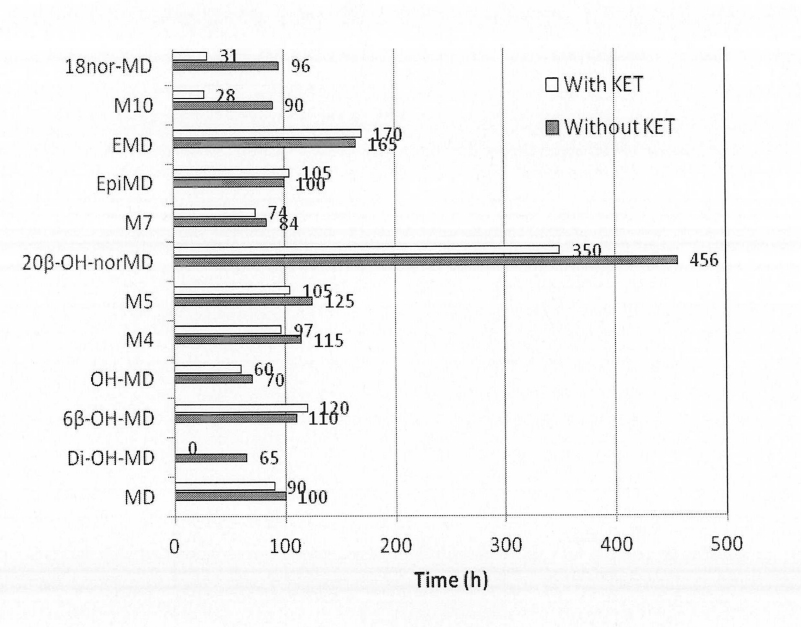

Figure 3: Windows of detection of methandienone and its metabolites after oral administration of 5 mg of methandienone (grey bars) and after combined administration of 5 mg of methandienone and 200 mg ketokonazole (white bars).

Development of a LC-MS/MS method to detect ketoconazole

The possibility to detect ketoconazole in the urine samples collected after the combined administration of methandienone and ketoconazole using the LC-MS/MS method routinely used in our laboratory to screen for methandienone and its metabolites was also investigated. After oral administration of a single dose of 200 mg, Ketoconazole is detectable in urine samples for at least seven days with a maximum of excretion in the range of 4-7 h from the oral administration (see Figure 4).

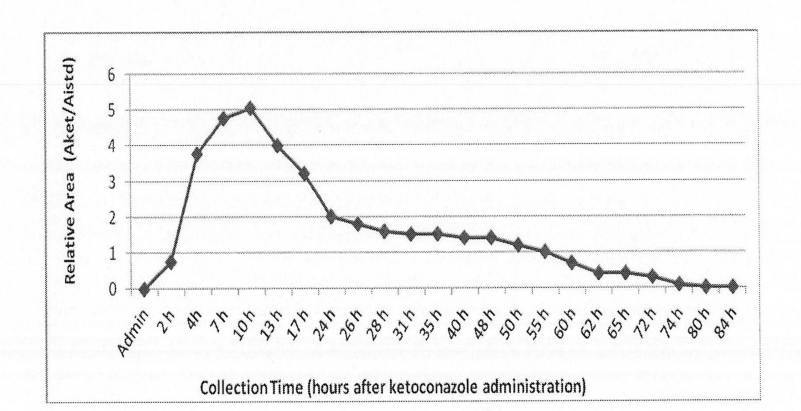

Figure 4. Urinary excretion profile of ketoconazole after oral administration of 200 mg of ketoconazole. The analytical procedures utilized to detect ketoconazole was those currently followed by our laboratory to detect methandienone and its metabolites.

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Conclusions

The data obtained show that inhibitory drug-drug interactions at the level of drug metabolism may occur between prohibited and non-prohibited substances causing an alterations of the levels, kinetics and windows of excretion of the metabolites selected as “diagnostic markers” of short- and long-term detection of the compounds banned for doping. If not identified, these effects – although weak in an absolute scale – may increase the risk of adulteration of analytical results. To increase the relevance of these observations, our next step is to evaluate the effect of other classes of banned substances, other CYP450 inhibitors and administration routes and to monitor the real occurrence of these not-prohibited agents in the urine samples collected in occasion of doping control tests.

References

[1] World Antidoping Agency. Laboratory statistics: http://www.wada-ama.org/Documents/Resources/Testing-Figures/WADA-2012-Anti-Doping-Testing-Figures-Report-EN.pdf
[2] Massé R, Bi H, Ayotte C, Du P, Gélinas H, Dugal R. (1991) Studies on anabolic steroids. The sequential reduction of methandienone and structurally related steroid A-ring substituants in human. A GC/MS study *J Chromatogr Biomed Appl* **562**, 323-340.
[3] Schänzer W. (1996) Metabolism of anabolic androgenic steroids *Clin Chem* **42**, 1001–1020.
[4] Schänzer W, Delahaut P, Geyer H, Machnik M, Horning S. (1996) Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography-high-resolution mass spectrometry. *J Chromatogr B Biomed Appl* **687**, 93–108.
[5] Schänzer W, Geyer H, Fußhöller G, Halatcheva N, Kohler M, Parr MK, Guddat S, Thomas A, Thevis M. (2006) Mass spectrometric identification and characterization of a new long-term metabolite of metandienone in human urine *Rapid Commun. Mass Spectrom*. **20**, 2252-2258.
[6] Lootens L, Meuleman P, Pozo OJ, Van Eenoo P, Leroux-Roels G, Delbeke FT. (2009) uPA^{+}/$^{+}$-SCID Mouse with Humanized Liver as a Modelfor In Vivo Metabolism of Exogenous Steroids: Methandienone as a Case Study *Clin. Chem.* **5**, 1783-1793.
[7] Pozo OJ, Lootens L, Van Eenoo P, Deventer K, Meuleman P, Leroux-Roels G, Parr MK, Schanzer W, Delbeke FT. (2009) Combination of liquid-chromatography tandem mass spectrometry in different scan modes with human and chimeric mouse urine for the study of steroid metabolism *Drug Testing and Analysis*, **11(1)**, 54-67.
[8] Zöllner A, Parr MK, Drăgan CA, Dräs S, Schlörer N, Peters FT, Maurer HH, Schänzer W, Bureik M. (2010) CYP21-catalyzed production of the long-term urinary metandienone metabolite 17b-hydroxymethyl-17a-methyl-18-norandrosta-1,4,13-trien-3-one: a contribution to the fight against doping *Biol. Chem.* **391**, 119-127.
[9] Parr MK, Zöllner A, Fußhöller G, Opfermann G, Schlörer N, Zorio M, Bureik M, Schänzer W. (2012) Unexpected contribution of cytochrome P450 enzymes CYP11B2 and CYP21, as well as CYP3A4 in xenobiotic androgen elimination – Insights from metandienone metabolism *Toxicology Letters* **213**, 381-391.
[10] Fragkaki AG, Angelis YS, Tsantili-Kakoulidou A, Koupparis M, Georgakopoulos C. (2009) Statistical analysis of fragmentation patterns of electron ionization mass spectra of enolized-trimethylsilylated anabolic androgenic steroids *International Journal of Mass Spectrometry* **285**,58–69.
[11] Mazzarino M, Fiacco I, Palermo A, de la Torre X, Botrè F. The use of cytochrome P450 inhibitors in sport. A new generation of doping masking agents? In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent advances in doping analysis* (20), Köln.

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