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Metabolic study of Prostanozol using human liver microsomes and humanized mice

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Abstract

The first part of the study involved the clean up of the nutritional supplement called Orastan-E from Gaspari Nutrition. Analysis of the supplement Orastan-E content, after the clean up procedure, was performed by GC-MS and it indicated the presence of the anabolic steroid prostanozol. This could be confirmed by comparison with the commercially available pure reference standard of this steroid.
In the second part of this study the metabolism of the anabolic steroid prostanozol was investigated using human liver microsomes (*in vitro*) and a mouse model with a humanized liver (*in vivo*). Most steroids are subject to metabolic transformations in the human body to enhance their urinary excretion. Prostanozol was administered to uPA$^{+/+}$-SCID mice which harbour human hepatocytes in their liver and to non-chimeric mice (without transplanted human hepatocytes) as a control. Previously, it was shown that the humanized liver of these mice has functional phase I and II metabolizing enzymes. The extracted mouse urines were analysed with a GC-MS scan method and a LC-MS/MS precursor ion scanning method. The results of pre- and post-administration mouse urines were evaluated. In combination with the results of the microsomal *in vitro* incubations with the prostanozol reference standard, a complete overview of the prostanozol metabolism was obtained. A good comparison between the *in vitro* and *in vivo* technique was obtained for prostanozol metabolism, resulting in the detection of several metabolites which could be subdivided into 7 categories. In the future this combined approach can be helpful in the determination of steroid metabolism.

Introduction

To ensure adequate detection of steroid misuse in athlete's urine in the framework of doping control, knowledge about steroid metabolism is critical [1]. Determination of metabolites is ideally performed in human volunteers, however this is rather exceptional due to many side effects and the lack of pharmaceutical-grade availability of most steroids. The steroid metabolites, detectable in urine, are mainly produced by metabolic transformations performed by liver enzymes [1]. Therefore we utilized a chimeric mouse of which the liver was transplanted with functional primary hepatocytes of human origin. In combination with this animal model, also *in vitro* human liver microsomes were used.
Prostanozol has a 17β-hydroxy-5α-androstan-[3,2-c]-pyrazole structure [2]. The only difference with stanozolol is that it lacks a 17α-methyl group. It is available as reference standard, but can also be obtained as a nutritional supplement Orastan-E. In the past, some difficulties were encountered in the identification of steroid metabolites after administration of nutritional supplements [1]. The most ideal situation is to administer the pure reference standard. However these are not always commercially available, and in such case a purified compound of the nutritional supplements could offer a solution. Prostanozol reference standard is sold as an exogenous steroid linked with a THP (tetrahydropyranyl ether). Attempts in our lab to analyse prostanozol-THP dissolved in methanol failed, but detection of the prostanozol-THP was possible dissolved in ether (data not shown). However on WADA's Prohibited list prostanozol is referred to as the compound with the 17β-hydroxylgroup [2], so we also administered the commercial available ‘free’ prostanozol.
The aim of the study was to investigate the urinary metabolism profile of prostanozol via an *in vitro* and *in vivo* technique.

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Experimental

Reference material
Prostanozol (17β-hydroxy-5α-androst-2-eno-(3,2-c)-pyrazole) and Prostanozol-THP (17β-tetrahydropyranol-5α-androst-2-eno(3,2-c)-pyrazole) were purchased from Toronto Research Chemicals (TRC, Canada).
The steroid supplement Orastan-E was available on the web (Gaspari Nutrition, Lakewood, USA).

Clean up
For the clean up of the Orastan-E pills, SPE cartridges filled with silica gel were used. Elution solvents were based on the clean up protocol of Kazlauskas [3]. The content of approximately 5 pills of the supplement was homogenously mixed and brought upon the packed column. All the eluates were collected into separate tubes and evaporated, followed by derivatization and full scan GC-MS analysis. The SPE-fractions which contained prostanozol were dissolved in methanol, combined and evaporated. To administer these fractions to the mice, they were dissolved in 200 µL ethanol and 800 µL of oil.

In vivo excretion study
The project was approved by the Animal Ethics Committee of the Faculty of Medicine of Ghent University (ECD 06/09). The mice were produced as previously described by Meuleman et al. [4]. The supplement, the purified supplement and the reference standard were administered to the chimeric mouse model. As a control group also non-chimeric mice (mice of the same genetic background but not transplanted with human hepatocytes) were used to correctly estimate the contributions of either the mouse or human metabolism.
A separate suspension was made of the three different formulations of prostanozol for oral gavation to the mice, with an approximate single dose of 1 mg. The mouse urine was collected over 24 hours since the mice produce only a small amount of urine a day. The urine was collected via metabolic cages specially designed for small rodents (Tecniplast, Buguggiate Italy).

In vitro human liver microsomal incubation (HLM)
Only the reference standard of prostanozol was tested by use of HLM. In a first step prostanozol (40 µg/mL final concentration) was incubated with phosphate buffer and NADPH regenerating system for 5 min at 37°C. In the next step the pooled microsomes were added, followed by incubation (for the desired time period) at 37°C. Whenever the reaction was stopped (0, 1, 2, 3, 4, 5, 6 and 18h), 25 µL of perchloric acid was added, followed by centrifugation. Control samples were used to verify the enzymatic reactions. All reagents were purchased from BD Bioscience-Gentest (Erembodegem, Belgium).

Sample preparation and Instruments
Both the mouse urine (500 µL) and microsomal incubations (200 µL) were extracted using the same sample preparation protocol. Internal standard (17α-methyltestosterone) and phosphate buffer were added, together with β-glucuronidase for hydrolysis of the glucuronides. After heating for 2.5 h at 56°C, 5 mL of diethyl ether and carbonate buffer was added and this mixture was rolled for 20 min, followed by centrifugation. The organic layer was separated and evaporated. After TMS-derivatization (100 µL of MSTFA/Ethanethiol/NH_4I mixture) the extracts were analysed with a full scan GC-MS method (Agilent, Palo Alto, USA). For LC-MS/MS (Thermo, San Jose, USA) no derivatization is needed and after evaporation, the extracts were dissolved in mobile phase of water:methanol (70:30). A precursor ion scanning method based on the method developed by Pozo et al. [5] was used. The same procedure was used for the HLM, except for the hydrolysis step, since only enzymes for phase I metabolism were activated.

Results and Discussion

GC-MS

The supplement Orastan-E seemed to be relatively pure since the analysis showed that it only contains sugar based fillers, prostanozol and no other steroid contaminants. Nevertheless, the clean up of the supplement resulted in a more concentrated amount of the compound of interest. In general, the same groups of metabolites were observed after all three different and separate administration studies in the chimeric mouse (supplement, clean up of supplement and reference standard). The best results were obtained after administration of the pure reference standard.

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Also the HLM were only incubated with the pure reference standard. Therefore the results are here discussed in general after prostanozol administration. In Figure 1 a GC-MS chromatogram of prostanozol is presented. The prostanozol compound has a molecular weight of 314, which after TMS-derivatization results in a molecular ion at 458 *m/z*.
When pre- and post-administration urine of chimeric mice was compared, several metabolites could specifically be detected. A high degree of similarity with the control non-chimeric mice was observed, indicating that there is a good correlation with the proper mouse metabolic data for prostanozol. These results were confirmed by the *in vitro* incubations based on liver microsomes from human origin (see below). In total 14 compounds were detected after prostanozol administration to the chimeric mice: the parent prostanozol itself and 13 different metabolites (Figure 2). Based on their mass spectra, all detected compounds could be divided in 7 categories of metabolites of prostanozol, indicated as M1-M7.

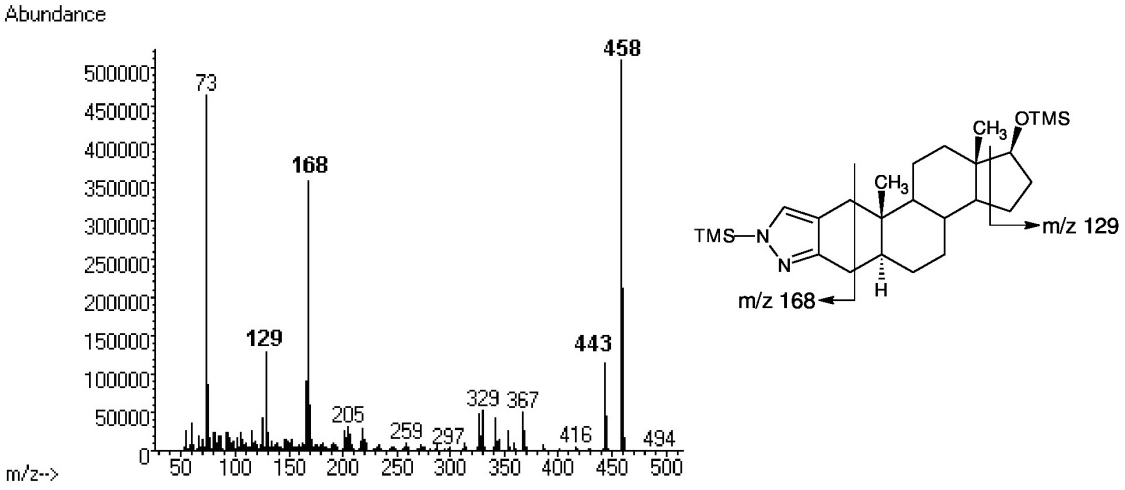

Figure 1: GC-MS mass spectrum of prostanozol (MW: 314; bisTMS: 458 m/z).

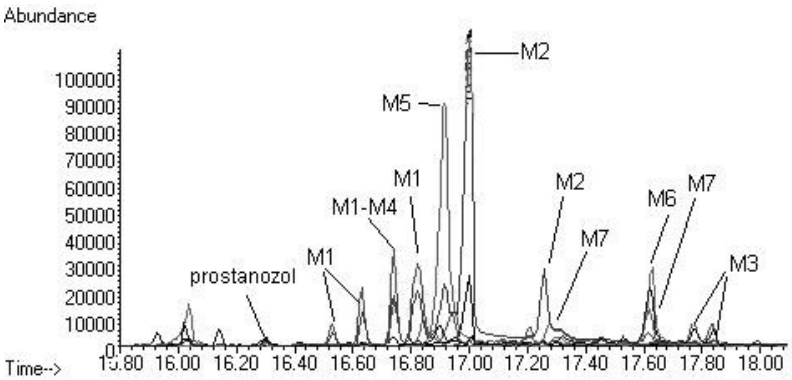

Figure 2: GC-MS extracted ion chromatogram of a 24h post-administration prostanozol chimeric mouse urine. The seven groups of metabolites (M1-M7) are indicated and are not present in the pre-administration urine. Several similar mass spectra were found for M1, M2, M3 and M7. The retention time of prostanozol itself is indicated; however other ions should be extracted. For mass spectra see Figure 3.

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Characteristic ions for the detection of prostanozol derivatives after trimethylsilylation (TMS) are the ions with m/z 254 and 168 (Figure 3). Fragment ion 254 *m/z* is an indicator for 3' or 4-hydroxylated metabolites of prostanozol, while ion 168 *m/z* gives an indication for metabolites having no hydroxylation in the A- or pyrazole ring [6-8]. The proposed positions of the hydroxyl-group, when *m/z* 168 is present, are C-6, C-12 or C-16, according to known hydroxylation positions in literature [9]. Since confirmation of the hydroxyl position is not possible solely based on the mass spectrum, they are indicated with 'x'. Within those 7 categories of metabolites, mono- and di-hydroxylated compounds were observed with a subdivision of possessing a hydroxyl group (M1-2-3-7) or a keto group (M4-5-6 and 17-ketoprostanozol) on the C-17. Some structural information could be derived from the mass spectra and based on that some tentative structure proposals were made (Figure 3).
Based on our results, M2 (546 *m/z*) and M5 (544 *m/z*) were observed as the most abundant, major metabolites. These metabolites are tentatively described as 17-ketoprostanozol and prostanozol compounds respectively, both with a mono-hydroxylation in the B-, C- or D-ring.

In literature only a few publications are available on human excretion studies with prostanozol [6-8]. Compared to the results obtained there, the same types of metabolites were found in our study using the mouse model and HLM, except for one metabolite. It was not possible to detect a metabolite with a dihydroxylated-17-keto-prostanozol structure (632/254 *m/z*), which was reported in humans. However, in addition using the mouse model and HLM, two new compounds were detected which were not previously described in literature: namely M1 and M7, proposed as a mono- and di-hydroxylated prostanozol metabolite respectively.

The results obtained after incubation of prostanozol reference standard with HLM indicated similar results. The same 7 groups of metabolites as previously described in the chimeric mice were present in the HLM incubations. The microsomal incubations confirmed metabolites of categories M1 and M7, which were not detected in previous human excretion studies.
Moreover one additional compound was present in the HLM incubations, namely 17-ketoprostanozol, which was previously only described in one of the human excretion studies [7]. Similar results were obtained with the different incubation periods; however 4 hours seemed to be the most optimal incubation time. The observations were compared with respect to the different control samples (no microsomes or no steroid present).

LC-MS/MS

The precursor ion scan method was performed to confirm that both 4OH as well as the 3'OH metabolites could be observed after prostanozol administration. On GC-MS it was not possible to unequivocally distinguish between those metabolites, without reference standards. For LC, three precursor ions (with indicated collision energy) are selected to have indications from the A-ring and pyrazole-ring: *m/z* 81 (45eV), 97 (45eV) and 145 (30eV). These ions were selected based on previous stanozolol research since the only difference is located in the D-ring compared to prostanozol [5]. Previous research demonstrated that fragment ion 81 *m/z* is an indication for an intact A- and pyrazole ring, while distinction of hydroxylation on the 3' or 4 position could be made by fragment ions *m/z* of 97 and 145 respectively (Figure 4). The biggest problem was to correlate the data obtained from GC- and LC-MS analysis. The results confirmed the presence of both 3'- and 4-hydroxylated metabolites.

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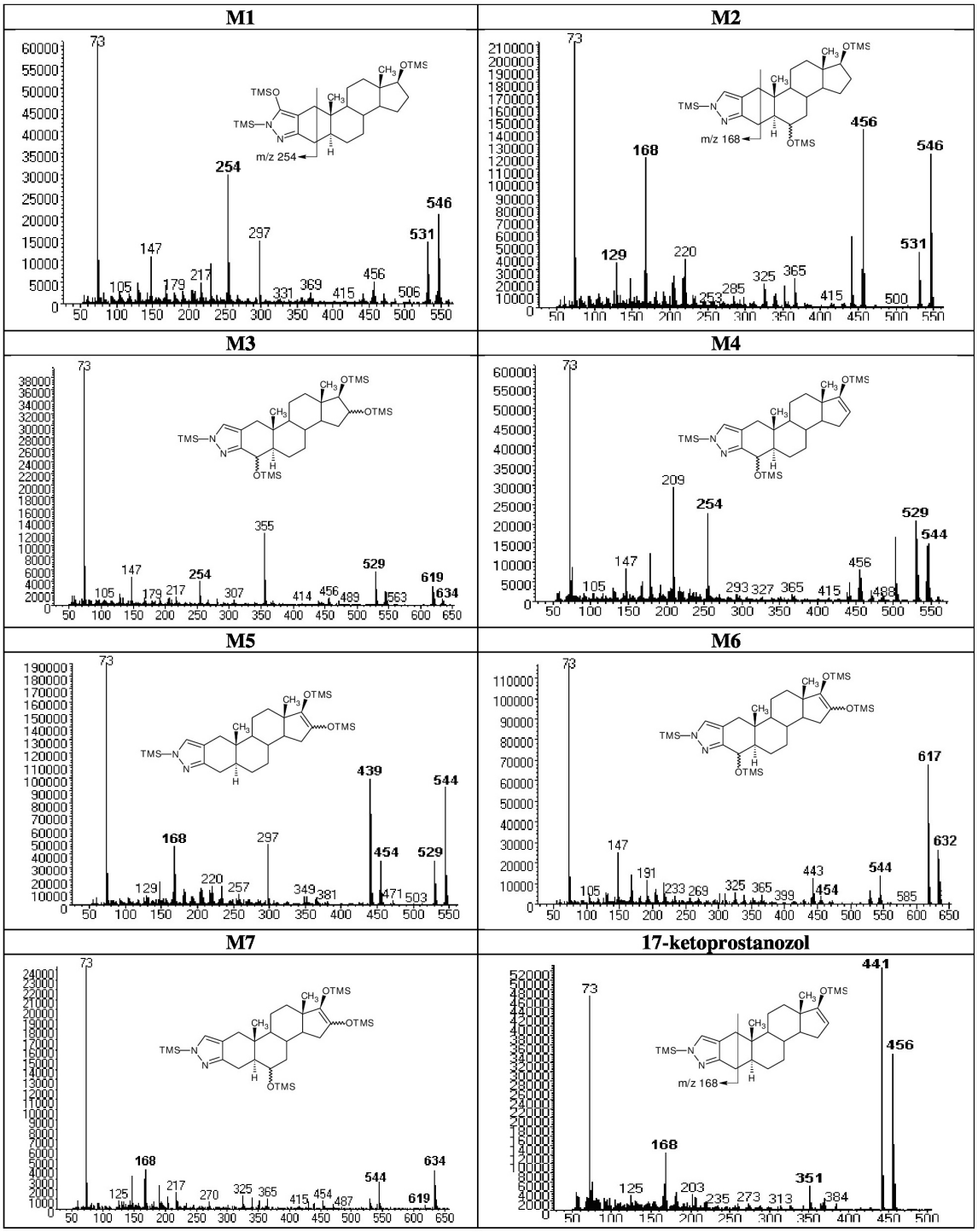

Figure 3: Mass spectra of the 7 categories of prostanozol metabolites and 17-ketoprostanozol detected in chimeric mouse and HLM studies, with tentative chemical structures. Legend: X-axis: m/z and Y-axis: Abundance.

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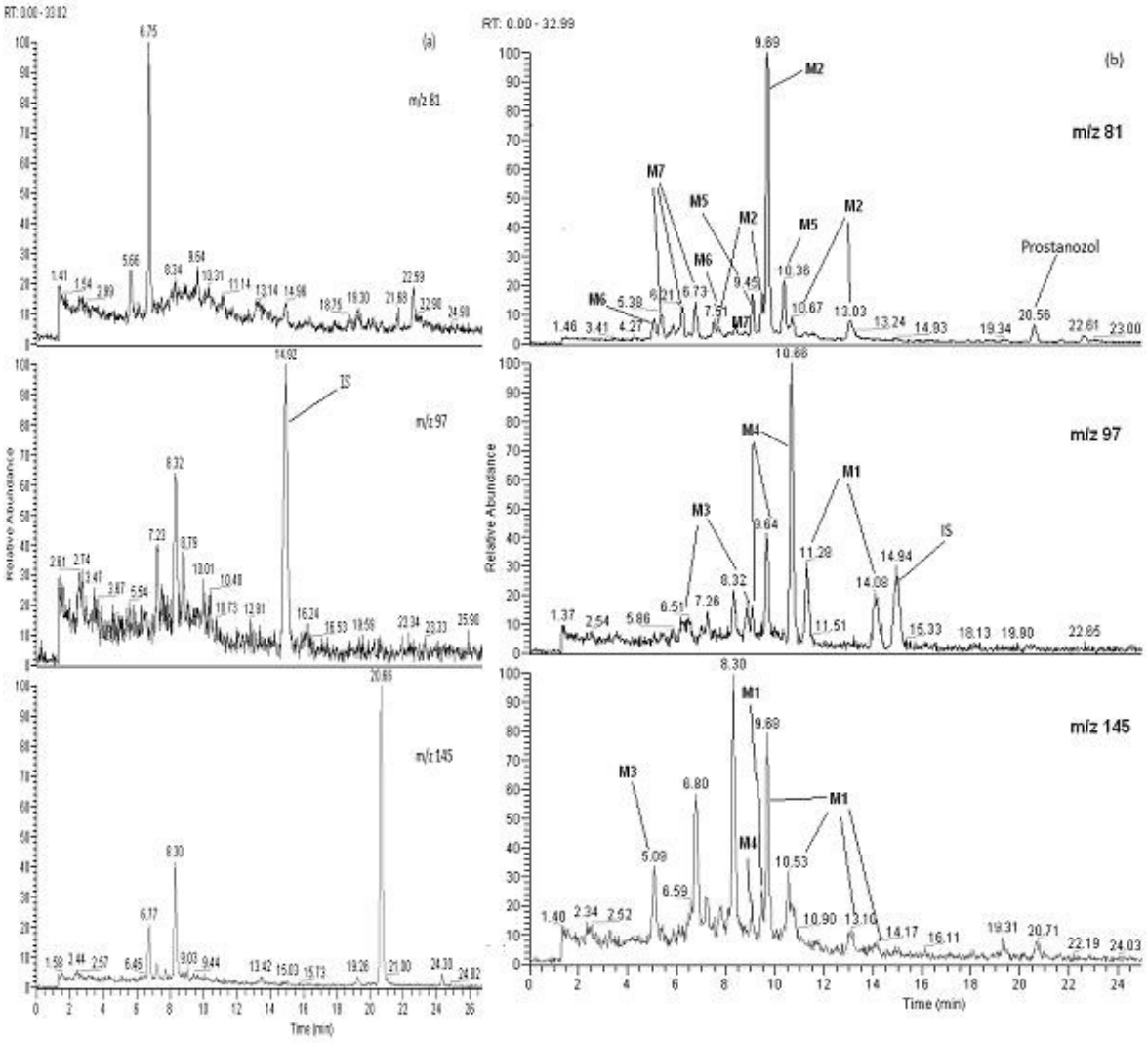

Figure 4: LC-MS/MS precursor ion scan chromatograms of (a) chimeric mouse urine collected before prostanozol administration and (b) chimeric mouse urine collected after prostanozol administration.

Conclusions

Orastan-E, a nutritional supplement, was cleaned up by use of SPE. The supplement contained the anabolic steroid prostanozol. The metabolism of prostanozol was investigated both via administration to chimeric mice with a humanized liver and via *in vitro* incubations with human liver microsomes. In total seven groups of metabolites were found by both techniques and one additional compound (17-ketoprostanozol) was found via HLM.
Using these models the detection of several prostanozol metabolites was possible; however for the unequivocal chemical structure elucidation and confirmation, reference standards are necessary. The results showed the comparability between the models with human excretion studies and hence provide further evidence for the applicability of chimeric mice and human liver microsomes to study metabolism of designer steroids.

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References

1. Lootens L, et al. (2011) Metabolic studies with promagnon, methylclostebol and methasterone in the uPA$^{+/+}$-SCID chimeric mice. *J Steroid Biochem Mol Biol* **127**, 374-381.
2. World Anti-Doping Agency. The 2012 Prohibited List. International Standard, Montreal (2012) http://wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2012/WADA_Prohibited_List_2012_EN.pdf
3. Kazlauskas R. (2010) ASDTL Supplements Project 2009. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (18),* Köln, pp 27-33.
4. Meuleman P, et al. (2005) Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology* **41**, 847-856.
5. Pozo O, et al. (2009) Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids* **74**, 837-852.
6. Kazlauskas R. (2006) Miscellaneous projects in sports drug testing at the National Measurement Institute. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (14),* Köln, pp 129-140.
7. Yum T, et al. (2011) Determination of metabolites of prostanozol in human urine by LC/ESI/MS and GC/TOF-MS. *Analytical Science and Technology* **24**, 173-182.
8. Rodchenkov G, et al. (2006) New designer anabolic steroids from internet. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (14),* Köln, pp 141-150.
9. Schänzer W. (1996) Metabolism of anabolic androgenic steroids. *Clin Chem* **42**, 1001-1020.

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