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Metabolic study of oxabolone using human liver microsomes and humanized mice

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Abstract

The urinary detection of oxabolone, a 19-nor anabolic steroid and potential doping agent, was investigated by evaluating its metabolic profile. Anabolic androgenic steroids comprise a popular class of performance enhancing products to be abused by athletes. The detection of oxabolone in urine in the framework of doping control is often based only on screening of the parent compound. However, it is well known that most steroids are metabolized in the human body. The liver is the key organ for these metabolic transformations. The formed metabolites are often longer detectable in urine than the parent compound itself. Moreover, the higher the number of markers are included in the initial testing procedure, the more evidence can be found of the illicit use of doping agents. Therefore oxabolone cypionate (esterified steroid) was administered to a chimeric uPA^{+/+}-SCID mouse model with a humanized liver. Additionally, oxabolone was also incubated with human liver microsomes. For the chimeric mouse model pre- and post-administration urine samples were collected over a 24 hour time period. For the *in vitro* incubations, the data within a time period of 18 hours were evaluated. Both analysis of the extracts of the chimeric mouse urine and the *in vitro* incubations were performed on GC-MS and on LC-MS, the latter by using precursor ion scan mode and high resolution. As a result, several oxabolone metabolites were detected by use of both models.

Introduction

Oxabolone is mentioned on the WADA Prohibited List [1] as an exogenous steroid and was formerly marketed as a pharmaceutical preparation Steranabol Ritardo[®] containing oxabolone cypionate [2]. It was developed for clinical purposes for the treatment of severe wasting disorders. This pharmaceutical preparation contained 25 mg of the oxabolone cypionate and formulation of the intramuscular preparation was aimed at sustained release of the compound from the site of injection. In the body the active steroid oxabolone is released from its esterified form. Oxabolone is a 19-nor-steroid with a hydroxyl function on the C4 position, which reduces its relative androgenicity and inhibits aromatization (Figure 1).

The oxabolone metabolism was already investigated in human in 1995 by Barbarulo et al. [2]. GC-MS was used as standard technique for the analysis of the post-administration urines of two male volunteers. However, nowadays more sophisticated and more sensitive techniques are available to reinvestigate oxabolone steroid metabolism. Besides the evolution of detection techniques, more strict legislation is hampering the use of human volunteers because of the side effects correlated with steroid use [3]. Therefore our lab has a chimeric mouse model with a functional humanized liver available as an alternative to investigate steroid metabolism [4]. This cannot replace human studies but in the past it has proven its value. Moreover, the combination with *in vitro* human liver microsomes (HLM) completes the strategy of investigation.

Experimental

Reference material

Oxabolone cypionate was available in the lab as an old lyophilized reference standard and oxabolone (4-hydroxy-estrene-17 β -ol-3-one) was available as methanolic solution from LGC Promochem. The latter was used for HLM, but not for

administration to the chimeric mouse model.

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 uPA^{+/+}-SCID chimeric mouse model was developed as previously described by Meuleman et al. [5]. Although the steroid ester preparation was originally made for injection, it was opted for oral administration of the steroid compound. The reference powder of oxabolone cypionate was dissolved in oil, since intramuscular injection of the chimeric mice requires a higher level of sterility related with their SCID background. Moreover it was already previously described that the route of administration does influence the absorption rate, but not the type of metabolites excreted in urine [6]. Based on this, the extensive hydrolysis step of the sample preparation and qualitative nature of the study, the effect of the administration can be approximated as non-significant.

Oxabolone cypionate (1 mg dose, single oral gavage) was administered to the chimeric mouse model (n=4). As a control group also non-chimeric mice (n=2), not transplanted with human hepatocytes but having the same background, were used to estimate the contributions of the proper mouse metabolism. The mouse urine was collected every 24 hours since the mice produce only a small amount of urine a day. The urine was collected separately from the faeces via metabolic cages from Tecniplast (Someren, The Netherlands) specially designed for small rodents. The 24h pre-administration urine was collected as 'blank' and the excretion urine was collected up to 48h post-administration.

In vitro human liver microsomal incubation (HLM)

At the first stage oxabolone cypionate and oxabolone (40 µg/mL final concentration) were incubated in different experiments with phosphate buffer and NADPH regenerating system for 5 min at 37°C. This was followed by adding the pooled microsomes and incubating in the Eppendorf tube (for the desired time period) at 37°C. To terminate the enzymatic reaction (0, 1, 2, 3, 4, 5, 7 and 18h), 25 µL of perchloric acid was added, followed by centrifugation. Control samples were used to verify enzymatically driven reactions. All reagents were purchased from BD Bioscience-Gentest (Erembodegem, Belgium).

Sample preparation and Instruments

The pre- and post-administration mouse urine and microsomal incubations were subject to the same sample preparation. First internal standard (17α-methyltestosterone) was added, together with β-glucuronidase for hydrolysis of the glucuronides. After heating for 2.5 hour at 56°C, 5 mL of diethyl ether and carbonate buffer is added to perform liquid liquid extraction by rolling for 20 min, followed by centrifugation. The organic layer was separated and evaporated. After TMS-derivatization (100 µL of MSTFA/Ethanol/NH₄I mixture) the extracts were analysed with a full scan GC-MS method (GC 6890N, MS 5975B from Agilent, Palo Alto, USA). For LC-MS/MS (HPLC Finnigan Surveyor MS with a Finnigan TSQ Quantum Discovery Max from Thermo, San Jose, USA) no derivatization is needed and after evaporation, the extracts were dissolved in mobile phase of water:methanol (70:30). 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

According to our results the cypionate group is cleaved off to liberate the active steroid oxabolone. Figure 1 represents the GC mass spectrum after tris-TMS-derivatization (m/z 506) of oxabolone after extraction from the 24h post-administration mouse urine. The loss of 15 amu, typical of steroids, resulting in m/z 491 is indicated. In the mouse model only a small amount of the administered esterified steroid could be detected (Figure 2, metabolite 16 corresponding to oxabolone cypionate).

The metabolic data obtained from the oxabolone cypionate 24h post-administration mouse urine revealed the presence of three major metabolites and several minor compounds (Figure 2). Each urine sample was analysed in triplicate to show the repeatability of the method. From the relative abundances it can be concluded that metabolite 8 (= 17-epimer of oxabolone), oxidized metabolite 9 (= 4-hydroxy-estr-4-ene-3,17-dione) and metabolite 10 (= oxabolone) are the three major metabolites. These results are partially confirmed in the literature where the 17-keto metabolite, corresponding to our metabolite 9, is described as the most abundant one in human [2,7].

However, only oxabolone and oxabolone cypionate, could be confirmed by direct comparison to reference standards available in the lab. The other compounds are not available as reference standard and identification is based on the mass spectra and the results from earlier studies [2,7].

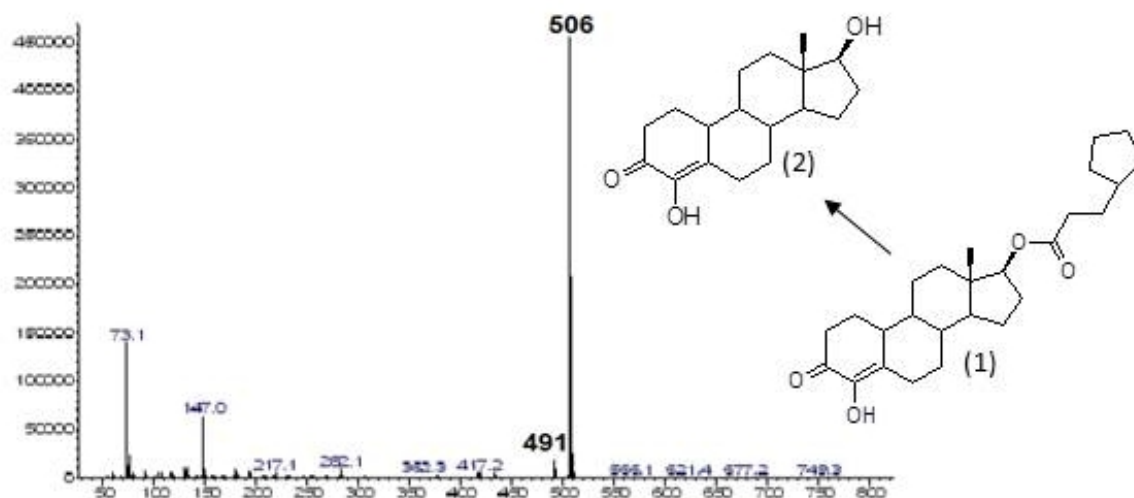


Figure 1: Chemical structures of oxabolone cypionate (1) and oxabolone (2). The GC chromatogram of oxabolone after TMS-derivatisation is shown (m/z 506).

When comparing the results from the chimeric and non-chimeric mice, the difference in abundances of the detected metabolites is remarkable especially with the same administered dose (Figure 2). The major difference between the chimeric and non-chimeric mouse can be seen in the relative abundance of the detected metabolites, indicating the contribution of the human hepatocytes in formation of the detected metabolites.

A schematic representation with potential structures of the detected metabolites was made based on the chimeric mouse excretion study (Figure 3). Direct cleavage of the ester group resulted in the detection of oxabolone and an epimer with equal abundances (m/z 506). Possible chemical structures for the other metabolites are indicated in Figure 3. The mouse model typically produces various isomers and here the metabolites can be categorized in four groups: (1) m/z 506, 3 minor metabolites with a possible reduction of 4-ene structure and oxidation of 17-hydroxy to 17-one functionality; (2) m/z 508, 4 metabolites possessing a completely reduced A-ring and 17-keto-functionality; (3) m/z 504, 2 compounds with oxidation e.g. to form a 17-keto group; (4) m/z 594, 4 mono-hydroxylated oxabolone metabolites (Figure 3).

Some of those metabolites were also reported in a previous publication of Barbarulo et al. [2]. However, some additional compounds like the hydroxylated metabolites with m/z 594 were detected in the mouse model and were not previously described in human. The current research suggests the extension of routine screening to target oxabolone, epi-oxabolone and 4-hydroxy-estrenedione and not only the parent compound.

Similar GC-MS results were obtained using the incubations with the HLM, both with oxabolone cypionate and oxabolone. The most representative results were obtained in the 18 hours incubation time point. However, for the chimeric mouse model the observed correlation was better with human metabolism than with HLM assay. Since more material is available from the HLM, also LC-HRMS experiments could be performed. This revealed the presence of a new compound (Figure 4). The accurate mass of this compound could be defined as m/z 312.25297 representing the ammonium adduct. A structure could be proposed for this compound which corresponds to a fully reduced metabolite with MW of m/z 294 (Figure 4). This compound would correspond on GC-MS with m/z 510. This was originally not detected, but when fractions were collected, this compound was seen although in very small amounts. Further research is necessary to confirm this experiment; however despite of the low proton affinity, this compound would be better screened on LC-MS-instruments as ammonium adducts.

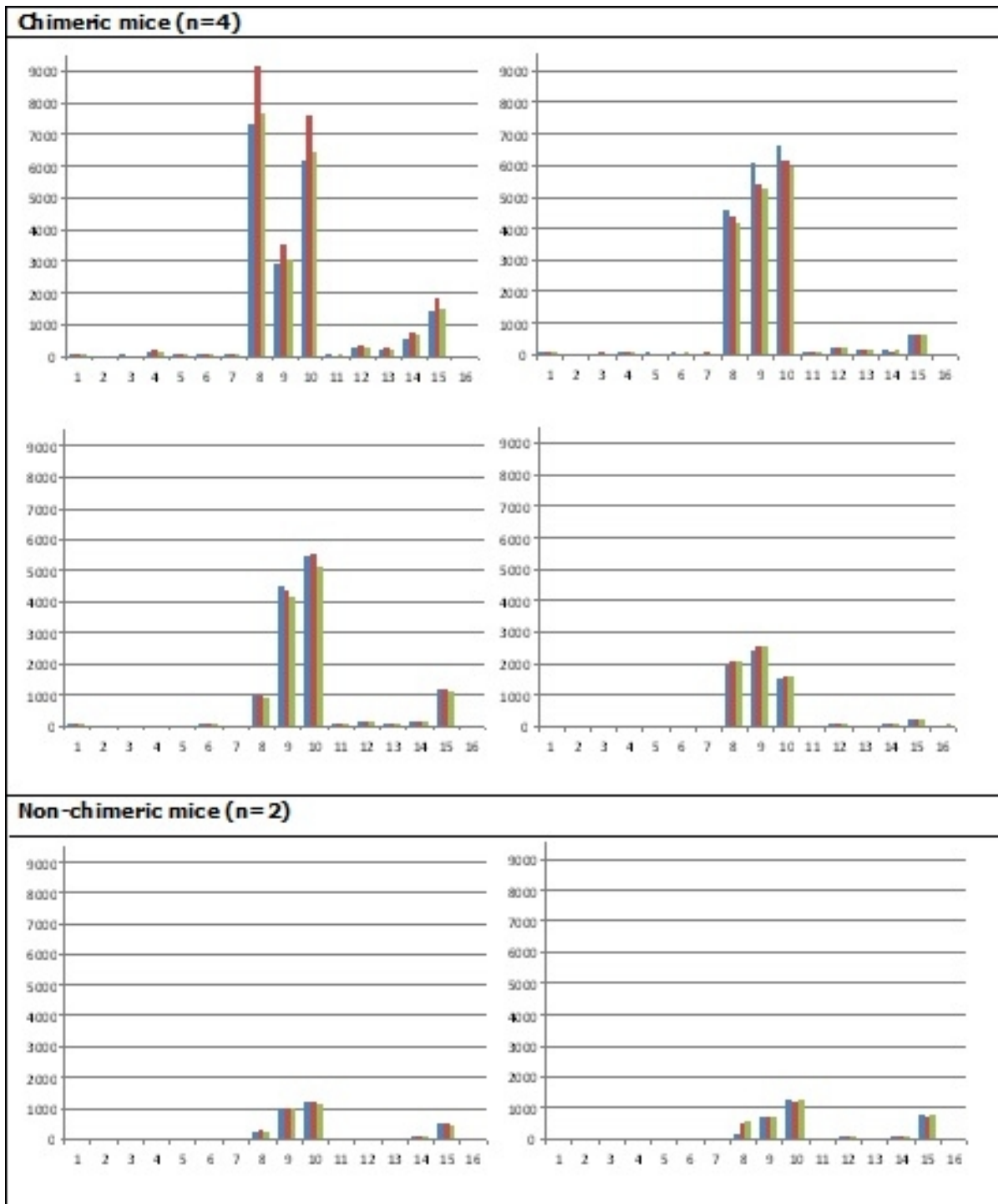


Figure 2: Oxabolone cypionate 24 hours post-administration urine of chimeric (n=4) and non-chimeric (n=2) mice. The relative abundances (Y-axis) of the detected compounds (1-16) are listed according to their retention time (X-axis). Each urine sample was analysed in triplicate (blue/red/green), to study the repeatability of the assay. Tentative assignments: 1-6-7: 4-OH-estrane-17-one, 2-3-4-5: 3,4-di-OH-estrane-17-one, 8: epimer of oxabolone, 9-11: 4-OH-estrane-17-one, 10: oxabolone, 12-13-14-15: mono-hydroxylated metabolites, 16: oxabolone cypionate.

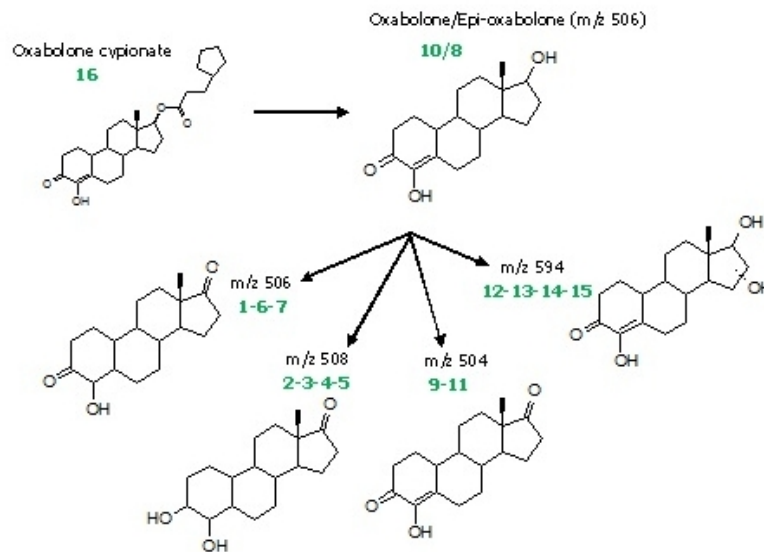


Figure 3: Schematic representation of the detected oxabolone cypionate metabolites categorized in 4 groups: Oxabolone, and proposed metabolites with MW of TMS derivatives of m/z 506, m/z 508, m/z 504 and m/z 594. The metabolites numbered in Figure 2 (1-16) are correlated with the potential structures, however these structure assignments are only a proposal.

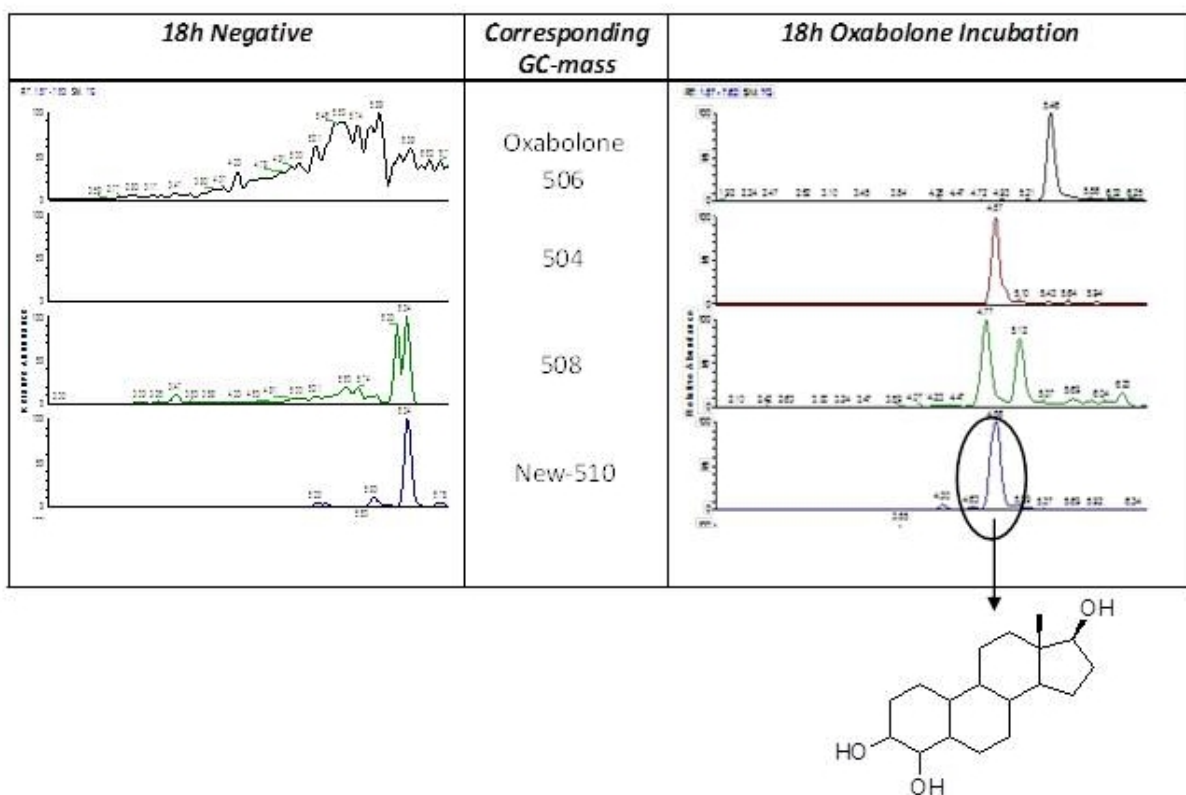


Figure 4: LC-HRMS results of the HLM incubations with oxabolone based on the comparison of a negative control and 18h incubation. The 'new' fully reduced compound with possible structure proposal is presented.

Conclusions

The metabolism of oxabolone was investigated by use of the chimeric mouse model and HLM. The combination of this *in vivo* and *in vitro* technique was the base for the research of urinary steroid metabolites to enhance the screening in human for anti-doping control purposes. The previous reported metabolites, all 17-keto metabolites with some reductions in the A-ring, could be confirmed and two types of new metabolites were detected. Whenever real human urine samples are available, the relevance of these metabolites will be verified. For structure elucidation and appropriate identification of the compounds, reference material is needed.

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