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Mibolerone Metabolism in the uPA$^{+/+}$-SCID chimeric mice

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Abstract

In the past, the chimeric mouse model has proven to be an excellent model to perform *in vivo* metabolic studies of anabolic steroids. The chimeric model consists of a transgenic uPA$^{+/+}$-SCID mouse transplanted with functional primary human hepatocytes. A useful application of the chimeric mouse model is to reinvestigate the metabolism of known steroids.
Mibolerone, mentioned on the WADA-prohibited list, was selected to perform an *in vivo* excretion study with the chimeric mice. For ethical reasons, this *in vivo* humanized animal model is more accessible than human volunteers to perform metabolic studies of anabolic steroids, since steroid use is often associated with serious side effects like liver toxicity.
Mibolerone was administered to 3 chimeric mice having different degrees of chimerism and to 1 non-chimeric mouse (without human hepatocytes) as a control. Excreted urine was collected after 24 hours via special designed metabolic cages for small rodents. The results of pre- and post-administration mouse urine, after analysis with a GC-MS scan method and a LC-MS/MS open screenings method, were evaluated and discussed.

Introduction

For the purpose of anti-doping screening, detection of steroid metabolites in urine is necessary. The market of steroids is evolving continuously due to ethical concerns and the illegal production of testosterone-derivatives (for non-therapeutic use) in order to escape from the control mechanisms. Although the use of steroids leads to some severe adverse side effects, they are still popular because of their performance enhancing effects, like muscle growth and recuperation capacity [1]. Mibolerone, which is mentioned on the WADA-prohibited list, is a 17-methylated exogenous steroid with an estrane core and an additional methyl group on the $C_{7\alpha}$-position (Figure 1) [2].

Mibolerone was already synthesized in the 1960' as a veterinary medicine for female cats and dogs controlling their oestrous cycle. For human use, some clinical studies have been performed. Although the pharmaceutical preparation was retracted from the market because of health risks, it was nevertheless quickly picked up by some athletes because of the acclaimed performance enhancing effects. Therefore a methodology for detection in human urine samples is necessary.

Since the *in vivo* chimeric 'humanized' mouse model is now available, it seemed appropriate to reinvestigate the metabolism of mibolerone.

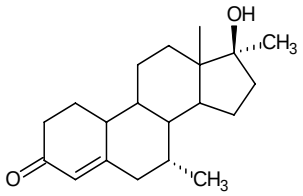


Figure 1: Chemical structure of mibolerone.

The chimeric mouse model has functional human phase I and phase II metabolizing enzymes, which are present in the liver and play an important role in steroid metabolism. The uPA$^{+/+}$-SCID mice feature two characteristics (induced liver disease and defective immune system) which create a perfect environment for transplantation with hepatocytes from human origin [3]. Since the liver is the main organ for metabolic transformations, this small animal model offers a perfect ethically better accepted alternative to perform steroid excretion studies than compared with humans or most *in vitro* techniques. The study of the metabolism of steroids is necessary since in most cases only small amounts of the parent compound are detected, while the detection of metabolites can assure a longer detection period of the abused doping products.

In literature only a few publications describing mibolerone metabolism in humans were found [4,5,6]. Besides the parent, the so called tetrahydromibolerone, 7α,17α-dimethyl-5β-estrane-3α,17β-diol, was proposed as the major metabolite. Only Zhang et al. [6] reported also one other di-hydroxylated metabolite.

In the study described herein, the results are discussed of the mibolerone administration study in the chimeric mouse model.

Materials and Methods

Administration study

The reference standard of mibolerone was a kind gift from Upjohn (Michigan, USA). For mibolerone administration to the mice, a suspension was made by dissolving the reference standard of mibolerone in methanol (10%), ethanol (20%) and further diluted with PBS to a final concentration of 50 mg/mL. From this suspension 150 µL was administered to chimeric (n=3) and non-chimeric (n=1) mice. The non-chimeric mouse is not transplanted and was used as a control to compensate for interspecies differences. From the chimeric mice, the human albumin concentration was measured in the mouse plasma as a marker for the level of replacement with human hepatocytes [3]. Mouse urine could be collected by using metabolic cages which separate the faeces and urine. Each 24-hour period the mouse urine was collected and stored at -20°C until the analysis. This project was approved by the Animal Ethics Committee of the Ghent University (ECD 06/09).

Analytical equipment

Sample preparation was based on the routine screening method for anabolic steroids. The only difference is that the extraction started from only 100 µL of mouse urine, because of the limited amount of daily produced mouse urine and the advantage of enabling replicate analysis [7]. As internal standard, 17α-methyltestosterone, with the same molecular weight as mibolerone, was used from Organon (Oss, The Netherlands).

After enzymatic hydrolysis with β-glucuronidase from *E. coli*, the steroid compounds are extracted from the urine with diethyl ether. After evaporation and TMS-derivatisation the samples were analysed first on GC-MS. The method was performed in full scan with mass range 50-800 *m/z*. The temperature program was as follows: initial temperature at 120°C, 60°C/min ->183°C, 3°C/min -> 232°C, 40°C/min ->310°C, with a total runtime of 22.33 min. To confirm the GC-MS results, some of the samples were once again extracted, followed by analysis with the precursor ion scanning method on LC-MS/MS [8].

Additionally the free and conjugated (glucuro- and sulpho-conjugated) fractions were investigated separately to test the distribution of the metabolites in the different fractions. The same urine was therefore analysed first without hydrolysis, followed by hydrolysis with β-glucuronidase from *E. coli* (release of glucuro-conjugates) and than by hydrolysis with β-glucuronidase from *H. pomatia* (release of sulpho-conjugates).

Results and Discussion

The mouse urine was analyzed and the data were processed by comparing pre- and post-administration urine samples. The pre-administration urine did not contain any steroid compounds, since the endogenous mouse steroid concentrations were too low to detect. Accordingly, all compounds detected in the 24h post-administration urine are derived from the mibolerone ingestion, confirming their metabolic nature (Figure 2). The results from one chimeric mouse, with the highest human albumin concentration, are discussed in detail since similar results were obtained for the other mice.

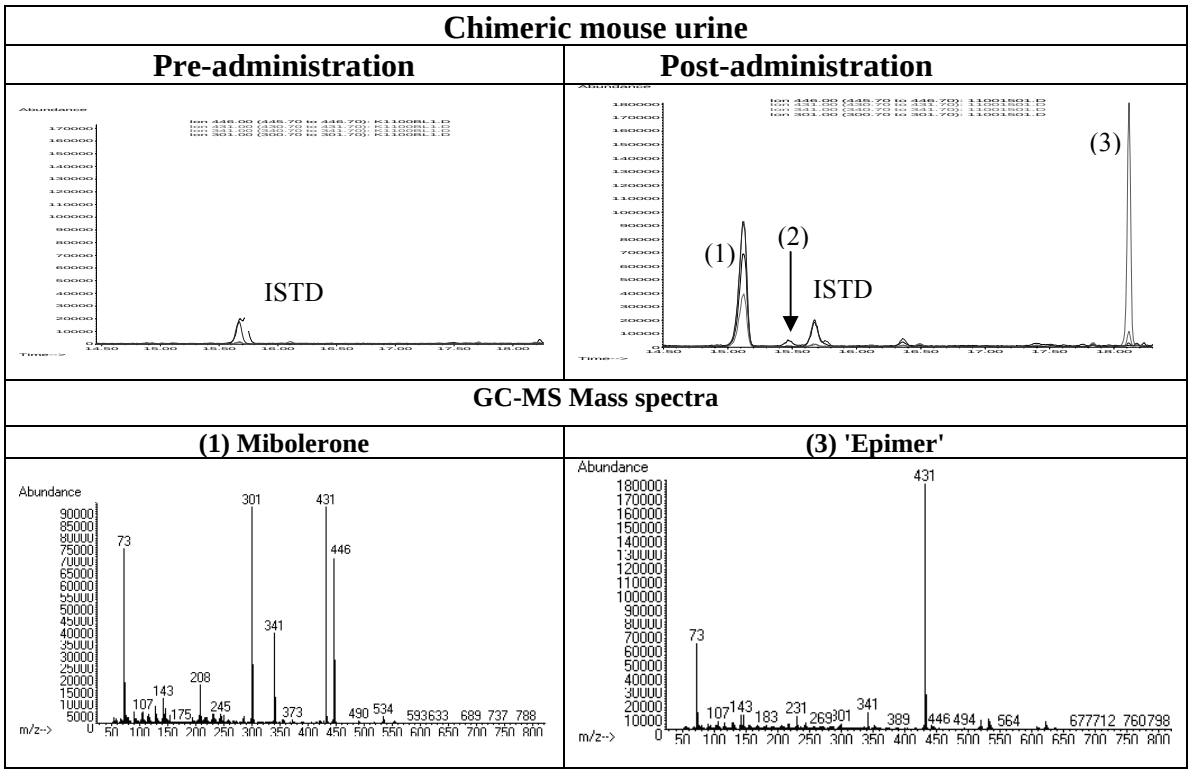


Figure 2: GC-MS extracted chromatogram of a 24 h mibolerone pre- and post-administration chimeric mouse urine, with the corresponding mass spectra of (1) and (3). Legend: ISTD: Internal Standard, 1= mibolerone, 2= unknown compound (see Figure 3), 3= 'epimer'.

The *bis*-TMS-derivatised parent mibolerone is detected at *m/z* 446. The mass spectrum of mibolerone is shown in Figure 2. Evaluation of the chromatogram presented in Figure 2, reveals besides the detection of the administered compound (1), also a major peak (3) with 431 *m/z* as most abundant similar ion in its mass spectrum, therefore called 'epimer'. Minor compound (2), in detail in Figure 3, is an unknown but interesting compound since it was not detectable in the non-chimeric control mouse. Until now, no additional structural information could be obtained.

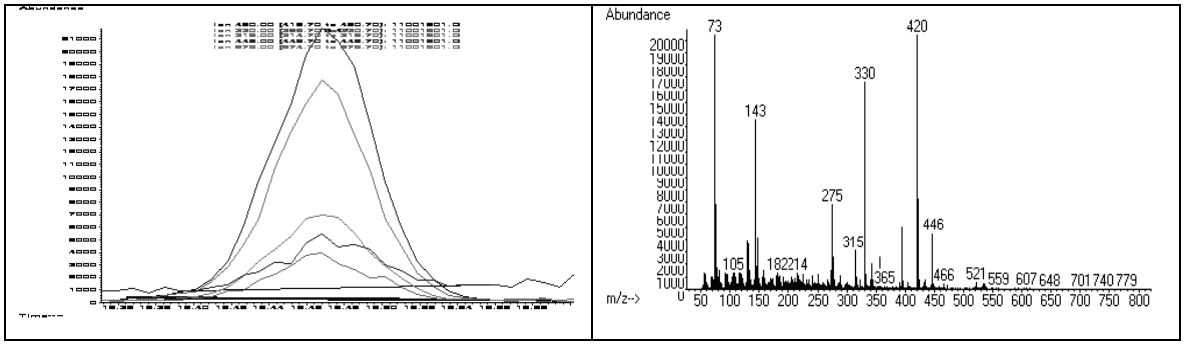


Figure 3: Extracted ion chromatogram of an unknown compound in post-administration chimeric mouse urine with its corresponding mass spectrum.

In order to differentiate between typical mouse and human metabolites, the post-administration data of a chimeric and non-chimeric mouse were also compared. A remarkable difference could be observed (Figure 4).

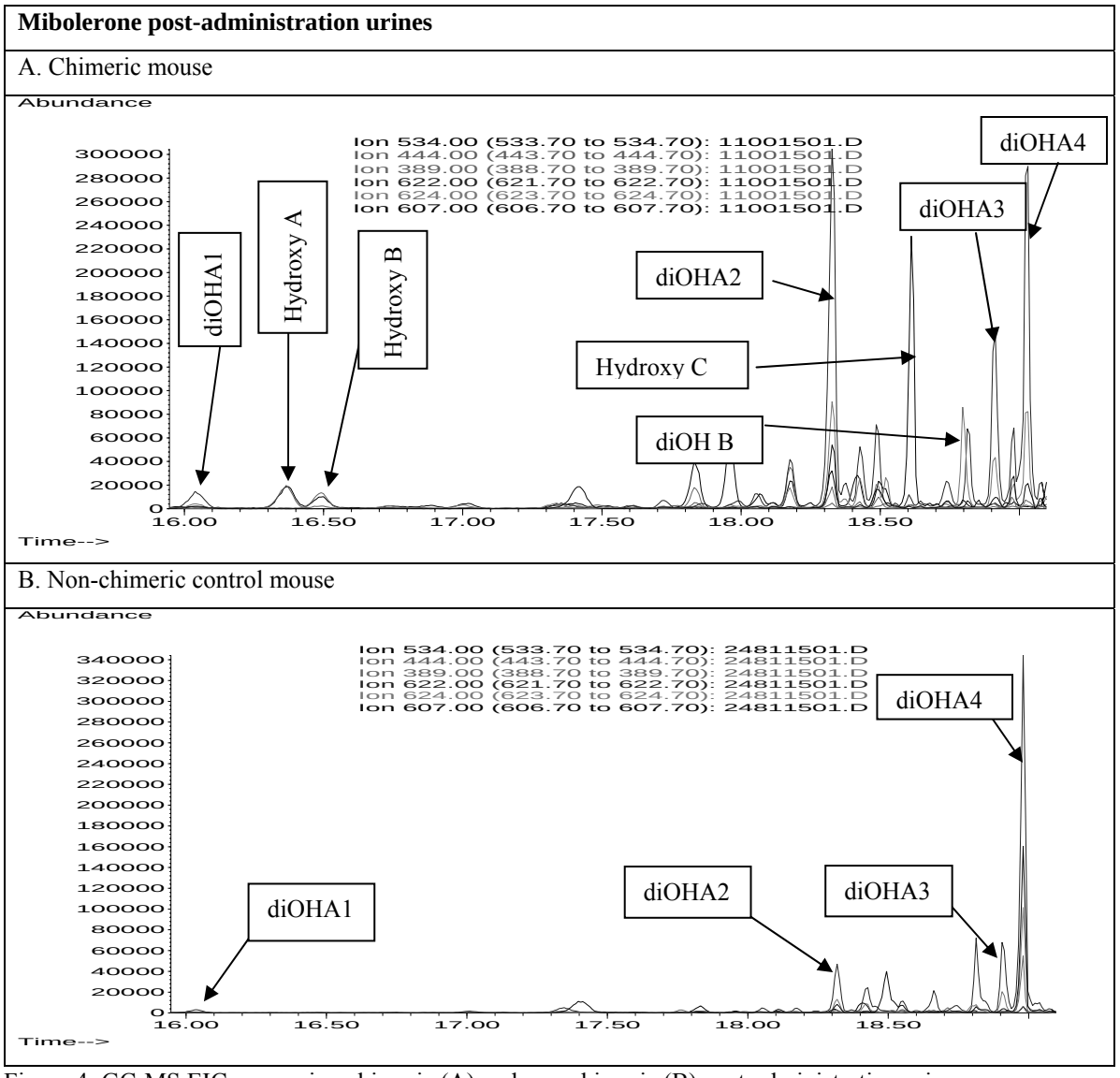


Figure 4: GC-MS EIC comparing chimeric (A) and non-chimeric (B) post-administration urine.

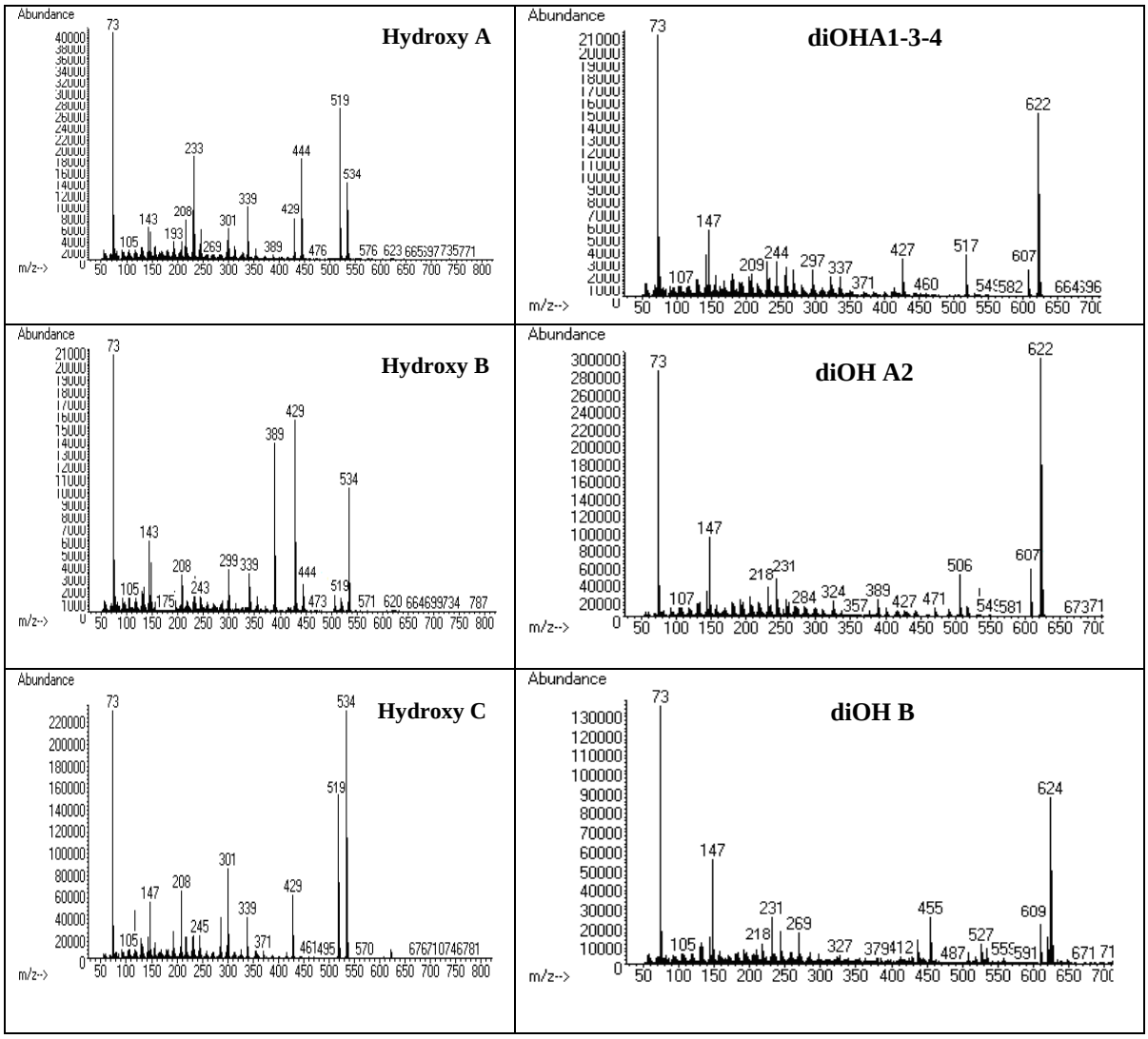


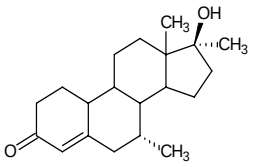
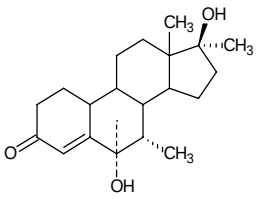
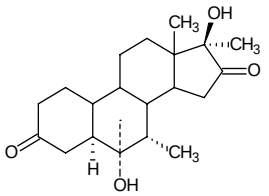
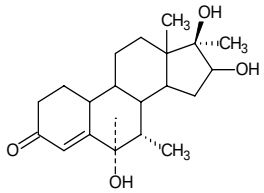
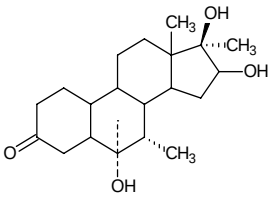
Figure 5: Mass spectra of the compounds (mono- and dihydroxylated) detected in the mibolerone post-administration chimeric mouse urine.

Three metabolites, named hydroxy A-B-C, have similar mass spectra, but small differences in their fragment ions (Figure 5-Table 1). The same theoretical mass of 534 *m/z* suggests the hydroxylation of mibolerone. The exact position of the hydroxyl group could not be assigned from the basis of the mass spectral data only.

Four dihydroxylated mibolerone compounds, diOH A1-A2-A3-A4, with a molecular weight of 622 *m/z* were detected. Depending on the position of the hydroxyl group, different mass spectra are obtained. For diOH A2 also ions 218 *m/z* and 231 *m/z* were present in the mass spectrum, typical for 16-hydroxylation [9]. The diOH B metabolite (MW= 624 *m/z*) corresponds theoretically with a dihydroxylated, but reduced mibolerone compound. This metabolite has strong similarities with data previously reported by Zhang et al. [6].

An overview of the detected metabolites is presented in Table 1 and Figure 6. Based on the mass spectrometric data, corresponding molecular weights and the knowledge about steroid metabolism in general, some tentative structures are proposed. Indeed, confirmation with a reference standard is still needed to unequivocally identify the detected metabolites.

Table 1: Overview of mibolerone metabolites detected in the chimeric mice.

Metabolites	***m/z***	**RT (min)**	**Tentative structure**
Mibolerone	446 431 341 301	15.12	
'Epimer '	431	18.13	**?**
Hydroxy A	534/519 444 429 339	16.37	
Hydroxy B	534/519 444 429 389	16.59	
Hydroxy C	534/519 429 339	18.62	
diOH A1, A3 en A4	622 607 517 244	A1: 16.04 A3: 18.91 A4: 19.03	
diOH A2	622 607 517 218/231	18.33	
diOH B	624 609 218/231	18.80	
Unknown compound	420 330	15.46	**?**

***Bolt:** compounds exclusively detectable in the humanized chimeric mouse, not detectable in the non-chimeric control mouse.

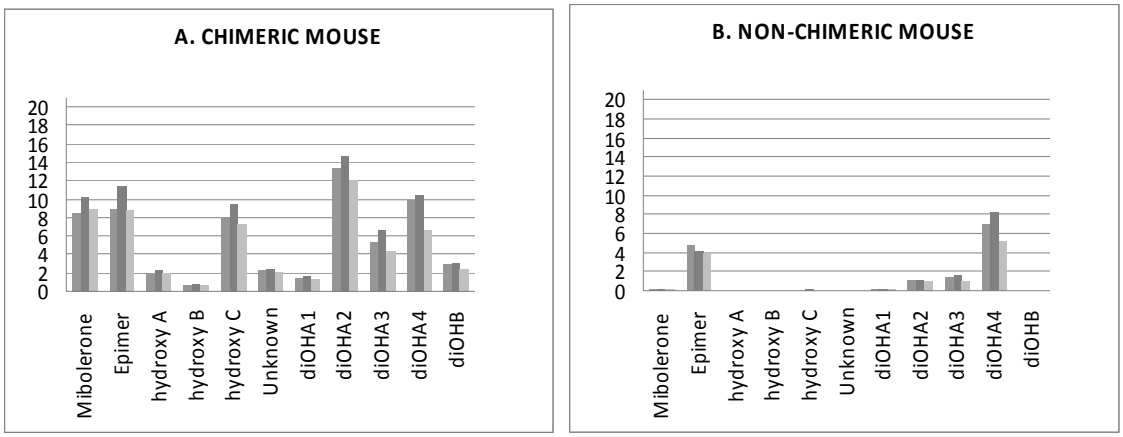


Figure 6: Graphical presentation of the detected compounds after mibolerone administration to one chimeric mouse and one non-chimeric (control) mouse. The analyses are performed in triplicate and are presented as relative abundances.

Some preliminary results on LC-MS/MS were also obtained in order to confirm data on GC-MS and to gather more, additional structural information about the compounds. It seems that most of the compounds were also detected on LC-MS/MS (Figure 7), however further research and evaluation of the data is necessary.

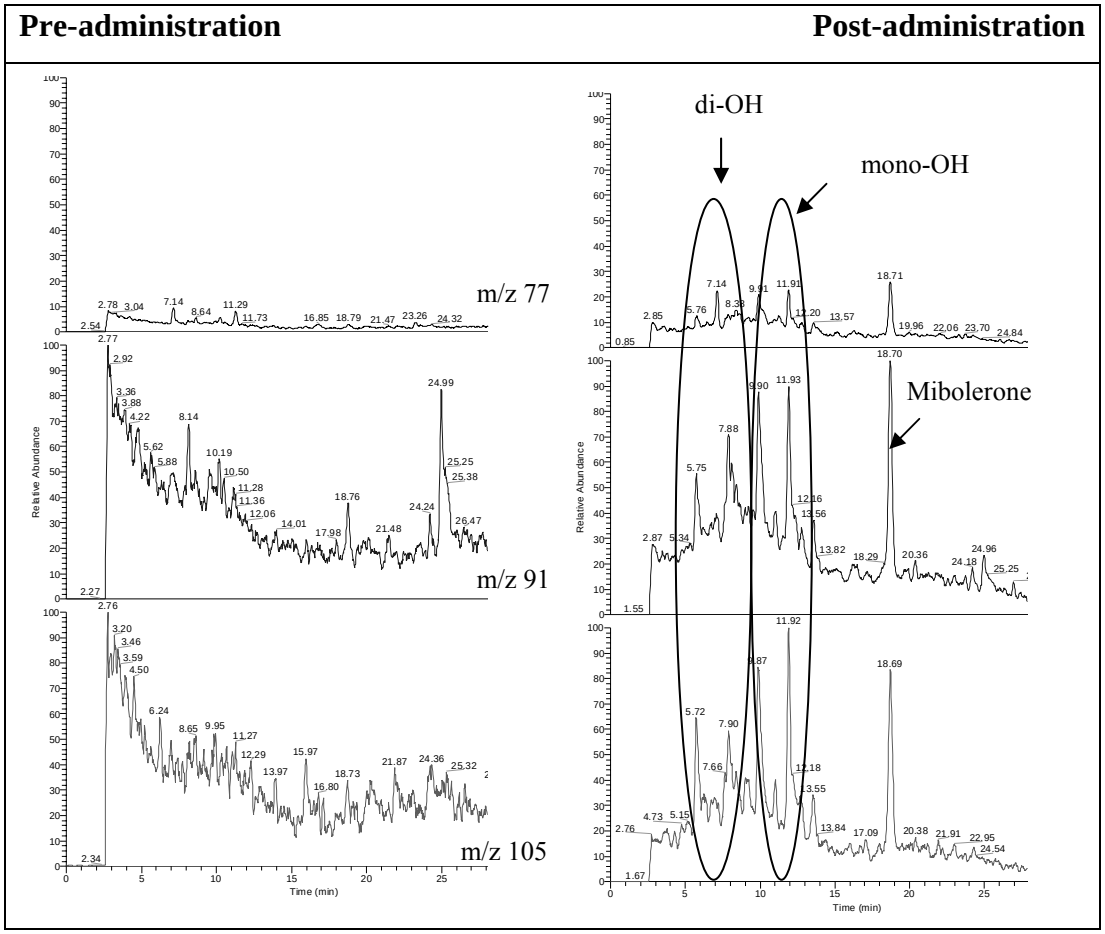


Figure 7: Preliminary LC-MS/MS results of mibolerone pre- and post-administration urine of a chimeric mouse urine.

All the results and figures discussed above are obtained by analysing the urine after enzymatic hydrolysis with β-glucuronidase from *E. coli*. Therefore the mibolerone metabolites were observed in the combined free and glucuronide-conjugated fraction, as it happens on a regular basis for routine doping screening. However to test the excretion profile of the metabolites, the urine was extracted in different steps to evaluate the unconjugated (free) and conjugated compounds separately. The study of the distribution of the metabolites showed that mibolerone itself, the mono-hydroxylated (hydroxyA-B-C), diOH B and the unknown compound were excreted mainly conjugated in the chimeric mouse urine, while the 'epimer' was excreted in unchanged form (free fraction). The di-hydroxylated metabolites (diOH A1-A2-A3-A4) were found both in the unconjugated and conjugated fraction (data not shown).

Conclusion

For the mibolerone excretion study, in total 11 compounds were detected in the post-administration mouse urine, 5 of which were exclusively detected in the chimeric mouse and not in the non-chimeric control mouse. The diOH B compound seemed to be previously reported in humans. Future experiments will include the incorporation of these metabolites in analytical methods to screen for human mibolerone misuse and production of reference standards via microsomal *in vitro* cultures.

Acknowledgements

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