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Validation of the metabolism of steroids in uPA^{+/+}-SCID mice with humanized liver

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Introduction

Recently a humanized mouse model has been developed for the study of viral hepatitis [1]. These mice suffer from a chronic liver disease caused by a liver specific over-expression of the uPA gene (urokinase-type Plasminogen Activator) and have a SCID (Severe Combined Immune Deficiency) background. This environment is optimal for transplanting the mice with healthy human hepatocytes and results in a chimeric mouse with restored normal liver functions. Meuleman et al. [1] confirmed the repopulation of the mouse liver with human hepatocytes via liver function tests and liver sections with specific staining.

Since anabolic androgenic steroids are mainly metabolised in the liver [2], doping control laboratories search for target compounds of the prohibited steroids in urine. Chimeric uPA^{+/+}-SCID mice could be used for the investigation of the metabolism of designer steroids or to confirm new metabolites of previously studied steroids. Hence, the humanized mouse could be an alternative for the suboptimal *in vitro* cultures or for the *in vivo* human excretion studies.

In a first step, the chimeric mouse model needs to be validated. Therefore 4-androstene-3,17-dione (AD) and metandienone (MTD) (Figure 1) have been selected as model compounds to compare metabolic pathways in humans and chimeric mice. The results of administration of AD and MTD to the mice are presented.

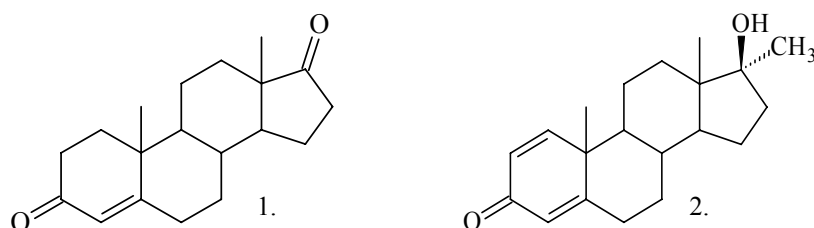


Figure 1: Chemical structures of (1) 4-androstene-3,17-dione (AD) and (2) metandienone (MTD).

Materials and Methods

1. Practical Design

Chimeric mice were produced as described before [1]. The mouse urine was collected using metabolic cages specially designed for small rodents (Tecniplast, Italy). The mice had free access to powdered food and water. Because of the design of the cages, urine and faeces are perfectly separated. The mice only produce an average of 1.5 mL of urine a day. uPA-SCID mice that were not transplanted with human hepatocytes served as a control group (= non-chimeric mice). The project was approved by the Animal Ethics Committee of the Faculty of Medicine of the Ghent University (ECD 06/09).

1.1 AD

The AD and placebo suspensions were administered to chimeric mice (n=6) and non-chimeric mice (n=4) via oral gavage. The 5% ethanol phosphate buffered saline (PBS) suspension of AD (3.5 mg/mL) was orally administered via a 100 μ L daily dose. The administered dose per kilogram bodyweight (bwt) was 10 times higher than the therapeutically advised dose in humans. Blank urine samples were collected 24 hours prior to dosage. In a double blind study protocol, AD or placebo suspensions were administered 3 times a week (Figure 2). The mouse urine was collected after a 24 h time interval and stored at -20°C awaiting analysis.

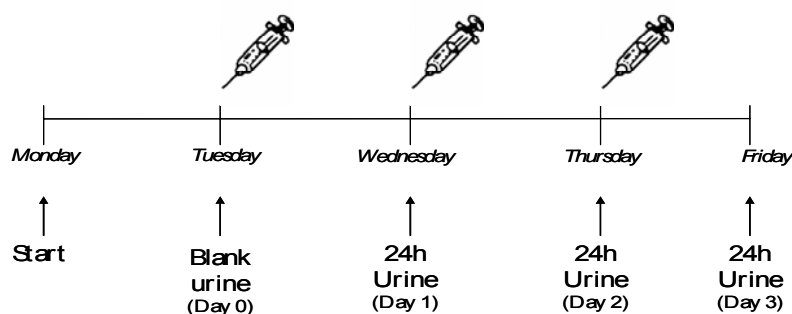


Figure 2: Gavation scheme of AD and/or placebo solution to the mice.

1.2 MTD

To a chimeric and non-chimeric mouse 100 μL of a placebo or a PBS-ethanol-MTD suspension (400 $\mu\text{g}/\text{mL}$) was administered. The MTD administered dose/kg bwt was 20 times higher than normally used in humans. The urine was collected every 24 h after administration. The results from the mice were compared with a database of 20 human urines previously declared positive for MTD.

2. Methods

The sample preparation and GC-MS method for the detection of AD and 15 other endogenous steroids in mouse urine was validated and previously published [3]. To obtain data on reproducibility, each analysis was done in triplicate.

An LC-MS/MS method was developed for MTD detection in mouse and human urine. The sample preparation was the same as for AD, except that for MTD 500 μL of urine was used and no derivatisation step was needed. The results of the method validation for the detection of MTD and its metabolites are submitted elsewhere [4].

Results and Discussion

1. Administration studies

1.1 AD

Preliminary results indicated that the steroid is cleared after 24 h, so multiple dosing was possible (Day 1-2-3). In the chimeric and non-chimeric mice no endogenous steroids were detected in the urine samples collected prior to administration and in the samples after placebo administration. The results obtained after AD administration to the mice, clearly show the differences between a chimeric and a non-chimeric mouse (Figure 3). In Figure 3.A the results of AD administration during 3 days to a chimeric and non-chimeric mouse are set relatively to the total amount of all steroids.

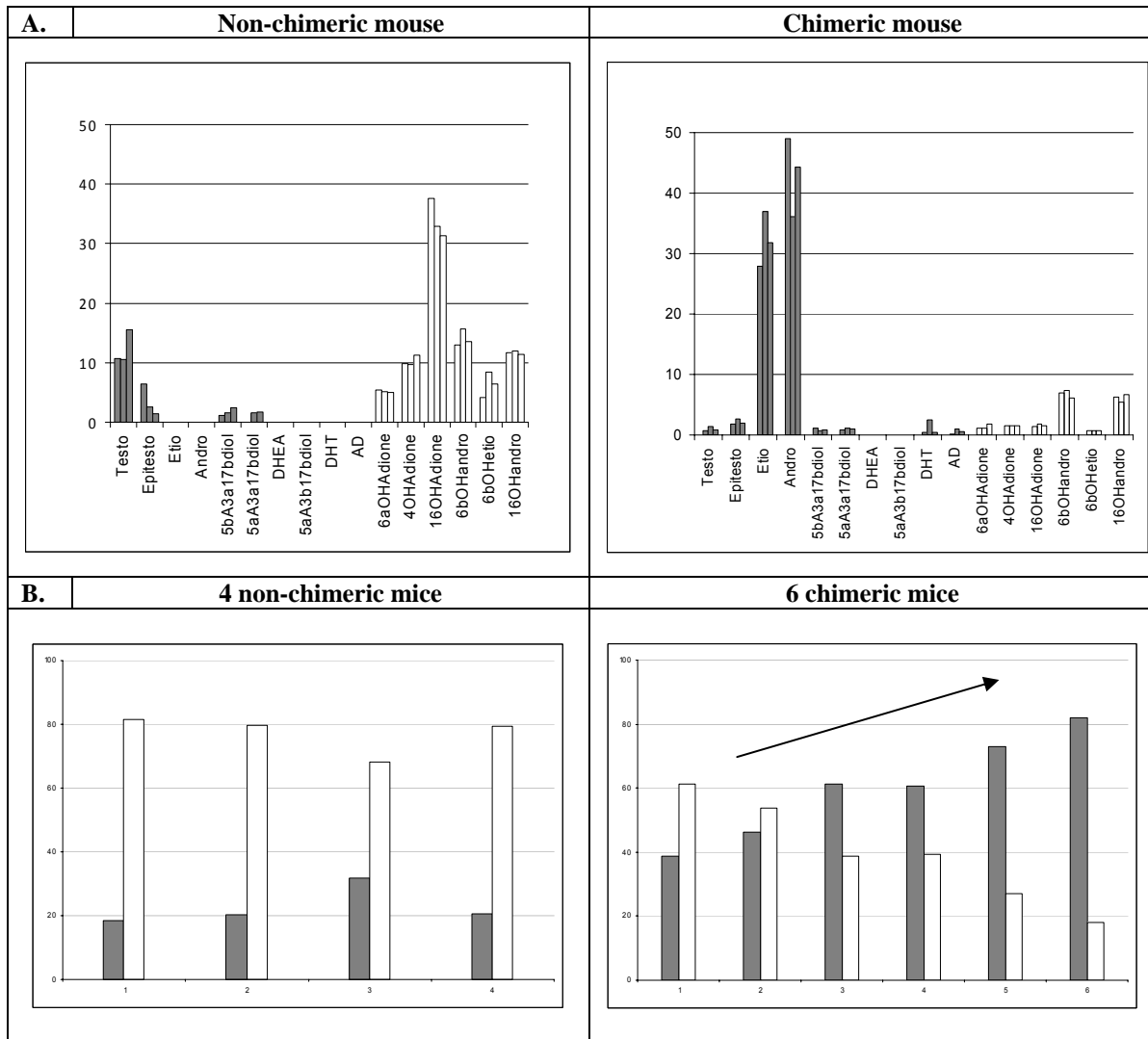


Figure 3:

A: Relative amounts of metabolites detected in the 24 h urine from a non-chimeric and a chimeric mouse after multiple AD administration over 3 days.

B: Results of AD administration to 4 non-chimeric mice and 6 chimeric mice with an increasing amount of human hepatocytes.

Legend

■ = **Human metabolites** = testosterone; epitestosterone; etiocholanolone; androsterone; 5 β -androstane-3 α ,17 β -diol; 5 α -androstane-3 α ,17 β -diol; dehydroepiandrosterone; 5 β -androstane-3 β ,17 β -diol; dihydrotestosterone; 4-androstene-3,17-dione.

□ = **Hydroxylated metabolites** = 6 α -hydroxyandrostenedione, 4-hydroxyandrostenedione, 16-hydroxyandrostenedione, 6 β -hydroxyandrosterone, 6 β -hydroxyetiocholanolone, 16-hydroxyandrosterone.

As shown in Figure 3.B the 16 steroids are divided in two groups, the human metabolites and the hydroxylated metabolites. As can be seen from Figure 3, hydroxylation is the major metabolic pathway in the non-chimeric control mice. These results from non-chimeric mice correlate with the findings reported from *in vitro* cultures, where hydroxylation is described as major metabolic pathway [5].

The main metabolites reported from excretion studies with AD in humans are androsterone and etiocholanolone [6]. These human metabolites were also detected in the chimeric mice (Figure 3).

AD was administered to 6 different mice with an increasing amount of human hepatocytes, based on the measurements of human albumin in the mouse plasma [1]. When analysing the urine of these 6 mice also an increase in human metabolites compared to the hydroxylated metabolites was observed (Figure 3.B).

1.2 MTD

The box and whisker plot in Figure 4 shows the distribution of the relative concentrations of 7 metabolites of MTD found in 20 real positive human urines. The major metabolite 6 β -hydroxymetandienone (6OH-MTD), detected in all human samples, was set as 100%.

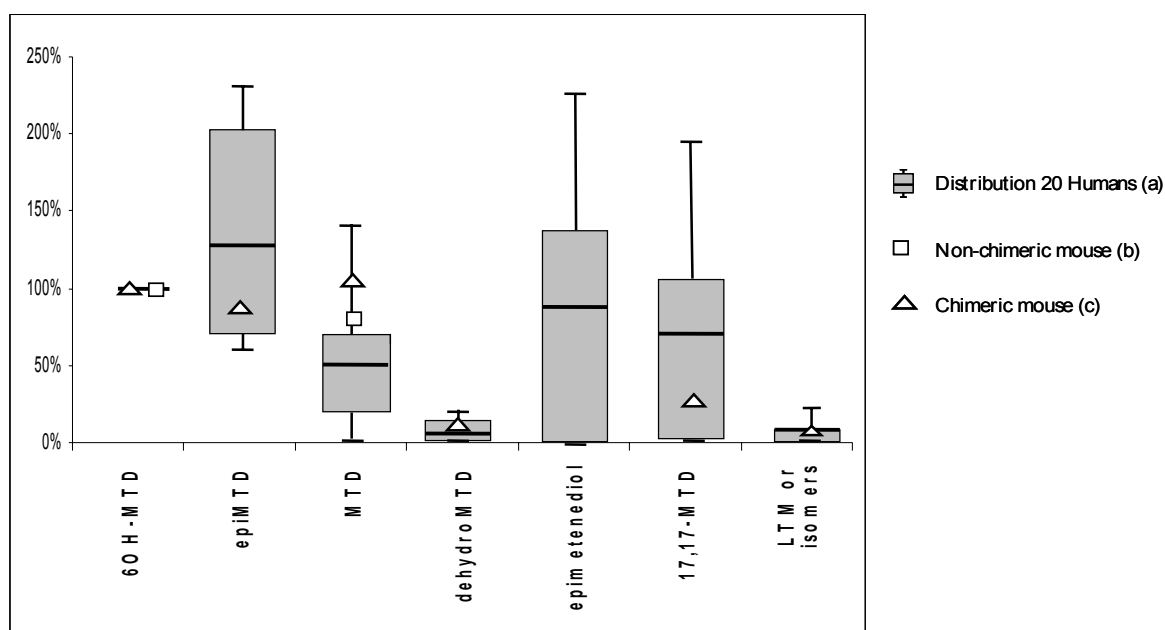


Figure 4: Box and whisker plot indicating (a) the distribution in 20 positive human urine samples for metandienone and the MTD metabolites detected in (b) non-chimeric mouse and (c) chimeric mouse urine after metandienone administration.

Legend: 6 β -hydroxymetandienone (6OH-MTD), 17-epimetandienone (epiMTD), metandienone (MTD), 17 α -methyl-17 β -hydroxy-5 β -androst-1-ene-3-one (dehydroMTD), 17 β -methyl-5 β -androstene-3 α ,17 α -diol (epimetenediol), 17,17-dimethyl-18-norandrost-1,13-dienol (17,17-MTD), 17 β -hydroxymethyl-17 α -methyl-18-norandrost-1,4,13-trienone (= Long Term Metabolite, LTM).

In the non-chimeric mouse only 2 out of the 7 compounds could be detected, i.e. MTD and its major metabolite 6OH-MTD. These results are similar to the findings previously reported by Kuuranne et al. [7] for *in vitro* cultures, showing 6 β -hydroxylation as the main metabolic

pathway.

In the chimeric mouse urine 6 out of the 7 compounds could be found, only epimetenediol was not detected. However, also in humans there seems to be a substantial variation in epimetenediol detection. It can not be excluded that epimetenediol is a minor metabolite in the chimeric mouse as well, but undetectable under the current circumstances. The LC-MS/MS chromatograms show the good correlation between humans and chimeric mice (Figure 5). Besides 6 β -hydroxylation, multiple metabolic pathways were found in the chimeric mouse including 17-epimerisation, 5 β -reduction and 3 α -dehydrogenation, similar to what was previously reported in humans [2].

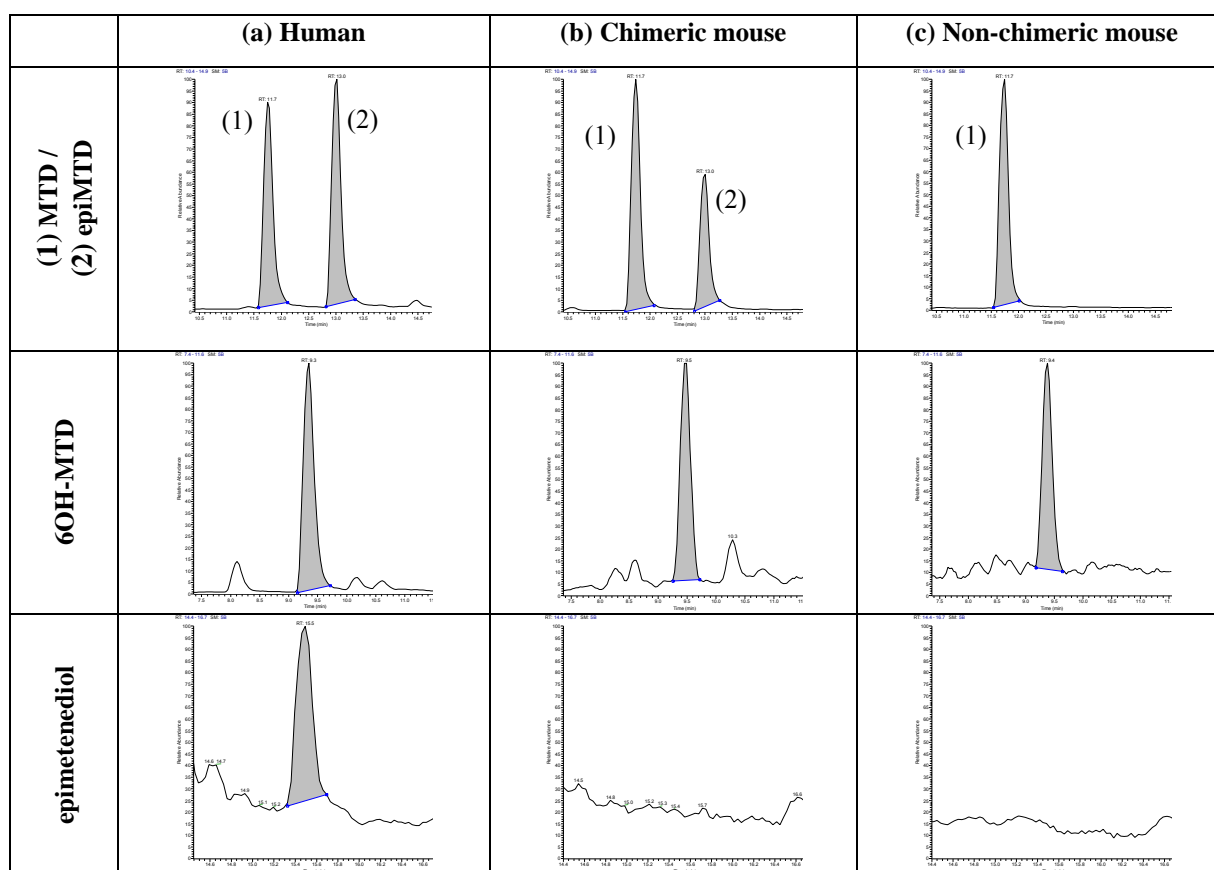


Figure 5: LC-MS/MS chromatograms for some MTD metabolites detected in (a) human, (b) chimeric mouse and (c) non-chimeric mouse urine samples.

Legend: (1) MTD; (2) epiMTD= 17-epimetandienone; 6OH-MTD= 6 β -hydroxymetandienone and epimetenediol.

Both types of mice also received a placebo solution. The analysis of these urines gave negative results, which indicates that the detected compounds are metabolites.

Conclusions

A chimeric uPA^{+/+}-SCID mouse model has been evaluated as an alternative for the *in vitro* and *in vivo* investigation of steroid metabolism. Studies with AD and MTD indicate the potential of the chimeric mouse as a model for human drug metabolism by showing parallel metabolic pathways. Based on the results, it seems that chimeric mice not only produce more metabolites than non-chimeric mice but also in the same relative amounts as in humans. Comparison with human data showed that chimeric mice have similar metabolic pathways as humans. The good correlation between chimeric uPA^{+/+}-SCID mice and humans indicates the applicability of the mouse model to confirm or even predict urinary metabolites.

Acknowledgements

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