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Steroid metabolism in chimeric mice with humanized liver

Leen Lootens,[a]* Peter Van Eenoo,[a] Philip Meuleman,[b] Oscar J. Pozo,[a] Pieter Van Renterghem,[a] Geert Leroux-Roels,[b] and Frans T. Delbeke[a]

Anabolic androgenic steroids are considered to be doping agents and are prohibited in sports. Their metabolism needs to be elucidated to allow for urinary detection by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS). Steroid metabolism was assessed using uPA$^{+/+}$ SCID mice with humanized livers (chimeric mice). This study presents the results of 19-norandrost-4-ene-3,17-dione (19-norAD) administration to these *in vivo* mice. As in humans, 19-norandrosterone and 19-noretiocholanolone are the major detectable metabolites of 19-norAD in the urine of chimeric mice.

A summary is given of the metabolic pathways found in chimeric mice after administration of three model steroid compounds (methandienone, androst-4-ene-3,17-dione and 19-norandrost-4-ene-3,17-dione). From these studies we can conclude that all major metabolic pathways for anabolic steroids in humans are present in the chimeric mouse. It is hoped that, in future, this promising chimeric mouse model might assist the discovery of new and possible longer detectable metabolites of (designer) steroids. **Copyright © 2009 John Wiley & Sons, Ltd.**

Keywords: steroids; metabolism; doping; chimeric mice; urine

Introduction

Steroids are among the most misused of the doping agents prohibited by the World Anti-Doping Agency (WADA).[1] The detection of steroid misuse by urine analysis requires a knowledge of steroid metabolism, which mainly occurs in the liver.[2–3] To obtain data on metabolism, human excretion studies are performed after oral steroid administration. Ethical approval is mandatory for these studies but it is not always easily obtained. Moreover, unlike registered drugs, most designer steroids, sometimes present in nutritional supplements, do not always undergo the appropriate quality tests.[4] Thus no information about their metabolism or toxicity is available. In most cases, *in vitro* studies or animal models are used to mimic the human metabolism.[5] However *in vitro* studies have several drawbacks, such as time limitations and the need of specific culture conditions (eg. enzymes and co-factors, which influence results.) Moreover, data obtained from animal models cannot simply be extrapolated to the human situation.

For these reasons we have evaluated a novel alternative chimeric mouse model for the *in vivo* study of steroid metabolism. This chimeric mouse model is based upon the transplantation of primary human hepatocytes in uPA-SCID mice that suffer from a transgene-induced liver disease.[6] Several weeks after transplantation, the livers of these mice will be largely occupied by functional human hepatocytes. These chimeric mice might therefore serve as a suitable alternative small-animal model for the *in vivo* investigation of steroid metabolism. The liver is the main organ for metabolization of xenobiotic compounds before excretion into the urine,[2–3] so this model with a humanized liver seems to be ideal for metabolic studies that would allow extrapolation of the data to humans. The chimeric mouse model was initially developed for the study of viral hepatitis.[6,7] So far, the only applications of the model in the pharmacology environment were studies on enzyme induction or inhibition.[3]

In the comprehensive study described here, the mouse model is used in the framework of doping control. The steroid metabolism in the chimeric model was evaluated by analysing the mouse urine after the administration of three selected steroids: 19-norandrost-4-ene-3,17-dione (19-norAD), androst-4-ene-3,17-dione (AD) and methandienone (MTD).

19-Norandrost-4-ene-3,17-dione (19-norAD), a prohormone of nandrolone (19-nortestosterone),[8] was introduced on the nutritional supplement market as a performance-enhancing agent. The metabolism of 19-norAD was previously studied *in vivo* in humans[8–11] and *in vitro*[12] with cultured primary human hepatocytes. Its administration in humans results in the formation of several metabolites, the major ones being 19-norandrosterone and 19-noretiocholanolone.

In this paper, the results of an administration study are presented in which gas chromatography-mass spectrometry (GC-MS) was used for the detection of 19-norAD and its metabolites in mouse urine. These results are compared with published data of human *in vivo* and *in vitro* studies.

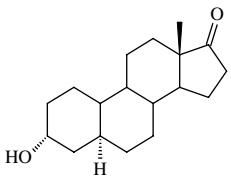
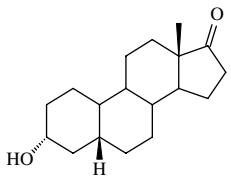
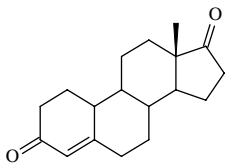
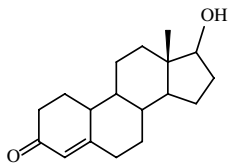
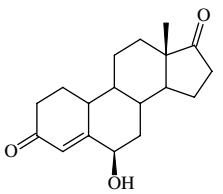
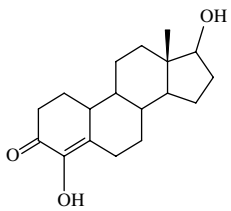
Previously, the metabolism of the two other steroids was evaluated in the chimeric mouse model, namely androst-4-ene-3,17-dione,[13] an endogenous steroid, and methandienone,[14] a 17-methylated steroid. A summary of these findings and an overall evaluation of the chimeric mouse model are also included in this paper.

* *Correspondence to: Leen Lootens, Docolab, Technologiepark 30, 9052 Zwijnaarde, Belgium. E-mail: leen.lootens@ugent.be*

a *Doping Control Laboratory (DoCoLab), Ghent University, Department of Clinical Chemistry, Microbiology and Immunology*

b *Center for Vaccinology, Ghent University and Hospital – Building A, first floor De Pintelaan 185, B-9000 Ghent, Belgium*

Table 1. Chemical structures of the monitored compounds and the GC relative retention times (RRT) with monitored m/z values in SIM-mode for 19-norandrost-4-ene-3,17-dione (19-norAD) and its metabolites after TMS-enol-TMS-ether derivatization

Compounds	Chemical structure	Monitored m/z values	RRT
19-norandrosterone		420, 405, 315	0.53
19-noretiocholanolone		420, 405, 315	0.59
19-norandrost-4-ene-3,17-dione (19-norAD)		416, 401, 194	0.74
Nandrolone (19-nortestosterone)		418, 403, 194	0.80
6β-hydroxynorandrostenedione		504, 489, 282	1.06
4-hydroxynandrolone		506, 491, 416	1.09

Experimental

Chemicals and reagents

19-Norandrost-4-ene-3,17-dione (19-norAD), nandrolone (19-nortestosterone), 4-hydroxynandrolone, 19-norandrosterone and 19-noretiocholanolone were purchased from the National Measurement Institute (NMI, Pymble, Australia). 6β-Hydroxynorandrostenedione was from Steraloids (Newport, USA). The internal standard, 17α-methyltestosterone, was a gift from Organon (Oss, The Netherlands). For structures see Table 1.

Phosphate-buffered saline (PBS) was from Invitrogen (Merelbeke, Belgium). Diethyl ether and ethanol were obtained from Biosolve (Valkenswaard, The Netherlands). Na_2SO_4, K_2CO_3 and NH_4I were purchased from Merck (Darmstadt, Germany). $NaHCO_3$ was from Fisher Scientific (Loughborough, UK). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Karl Bucher (Waldstetten, Germany), ethanethiol (97%) was from Acros (Geel, Belgium), β-glucuronidase from *Escherichia coli* K12 was from Roche Diagnostic (Mannheim, Germany) and β-glucuronidase from *Helix pomatia* from Sigma-Aldrich (Steinheim, Germany).

Methods

Study protocol

The project was approved by the Animal Ethics Committee of the Faculty of Medicine of the Ghent University (ECD 06/09). The mouse metabolic study was performed by oral gavation of a PBS suspension containing 19-norAD (350 µg/100 µL) or a placebo. The 19-norAD-PBS suspension was made by weighing pure reference

standard of 19-norAD, dissolving it in 5% ethanol and further diluting it with PBS to reach the final concentration. The placebo contained PBS with 5% ethanol.

Metabolic cages for small animal models were used (Tecniplast, Italy) to separate urine and faeces. Urine was collected every 24 h, followed by storage at −20 °C while waiting for analysis. Prior to administration blank urine was collected. Afterwards gavage was performed on days 1, 2 and 3 (D1-2-3). Between the administration of steroid and placebo there was a washout period of 72 h.

Animals

Chimeric mice (n = 6) and non-chimeric mice (n = 2) were used. In the metabolic cages the mice had free access to water and powdered food. The uPA$^{+/+}$-SCID chimeric mice were produced as described by Meuleman *et al.*[6,7] The amount of humanization of the chimeric liver was assessed by measuring the concentration of human albumin (hAlb) in the mouse plasma. The non-chimeric mice served as a control group to determine the metabolic pathways of murine hepatocytes.

Sample preparation

19-norAD and its metabolites were quantified by GC–MS (SIM) after hydrolysis. All urine samples were analysed in triplicate (n = 3). Briefly, the internal standard 17α-methyltestosterone, 1 mL of phosphate buffer (pH 7) and 50 µL β-glucuronidase from *E. coli* were added to 100 µL of mouse urine and the mixture was hydrolysed for 2.5 h at 56 °C. For the hydrolysis of sulphates 1 mL of acetic buffer (pH 5.2) and 50 µL of β-glucuronidase from *H pomatia* were added. After cooling to room temperature and adding $NaHCO_3/K_2CO_3$ (2/1; w/w) solid buffer, extraction was performed by rolling (20 min) with 5 mL freshly distilled diethyl ether. Once centrifuged, the organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated under oxygen-free nitrogen. The residue was derivatized with 100 µL MSTFA/NH_4I/ethanethiol (1280/3/5) for 2.5 h at 80 °C.

Gas chromatography-mass spectrometry parameters

The GC-MS analysis of the samples was conducted in the selected ion monitoring (SIM) mode on an Agilent 6890 gas chromatograph directly coupled to an Agilent 5975 mass selective detector (Palo Alto, USA). The GC-column was an HP-Ultra 1 (J&W, Folsom, CA, USA), 100% methyl silicone column with a length of 17 m, an internal diameter of 0.2 mm and a film thickness of 0.11 µm.

The oven temperature programme was as follows: 170 °C (0.5 min), 30 °C/min → 200 °C (0.2 min), 2 °C/min → 231 °C (0.1 min), 30 °C/min → 300 °C (1 min). The carrier gas was helium at a constant flow rate of 0.5 mL/min. The electron energy was set at 70 eV and the ion source temperature at 250 °C. Injection volume was 0.5 µL, splitless. For each compound three diagnostic m/z-values were monitored (Table 1).

Results and Discussion

Administration of 19-norAD

The major urinary markers of 19-norAD administration in humans are 19-norandrosterone and 19-noretiocholanolone. Minor metabolites such as 6β-hydroxynorandrostenedione and nandrolone have also been described.[8–11] Each of these four steroids was therefore included in the GC-MS method, which had been validated for quantitative purposes according to Eurachem guidelines.[15] The synthetic steroid 4-hydroxynandrolone (oxabolone) was also included.[9,16]

The mean urinary concentration (n = 3) per steroid after multiple 19-norAD administration (Day 1-2-3) in six chimeric mice and two non-chimeric mice is shown in Figure 1. The data are presented as relative amounts, excreted per detected steroid in each mouse. The variation between the three administration days (D1-D2-D3) can be related to uncertainty regarding the size of the administered dose of the 19-norAD suspension. Indeed, due to the low solubility of steroids in polar agents like PBS, a suspension was formed. Nevertheless, gavage of a PBS suspension was considered to be the best possible option for administering the steroids properly to the mice, because other administration routes (for example, mixed with feed) could lead either to contamination problems or to even higher variations in administered dose.

Placebo-blank

Both a placebo and the effective steroid suspension were administered to the mice via oral gavation. Analyses of the mouse urine after placebo administration did not result in the detection of 19-norAD or its metabolites. Likewise, the urine collected 24 h prior to the administration did not contain 19-norAD or any of its metabolic compounds (data not shown).

Non-chimeric mice

In the non-chimeric mice, four of the six monitored compounds were detected by GC-MS: 19-norAD, nandrolone, 6β-hydroxynorandrostenedione and 4-hydroxynandrolone (Figure 1). Neither 19-norandrosterone nor 19-noretiocholanolone were detected, although these metabolites are the major metabolites in humans and are regarded as the best urinary markers for misuse of nandrolone and its precursors (such as 19-norAD) in doping control.[9] On the other hand, a minor human metabolite, 6β-hydroxynorandrostenedione, was present in the non-chimeric mice (Figure 1). Despite the low absolute urinary concentration it should be noted that nandrolone, 6β-hydroxynorandrostenedione and 4-hydroxynandrolone were significantly more abundant in relative terms in non-chimeric mice compared to the chimeric mice. This relative predominance of hydroxylation pathways in the non-chimeric mice had already been noticed for AD[13] and MTD.[14]

Chimeric mice

The parent 19-norAD was almost completely metabolized, indicating that the metabolic enzymes are sufficiently present and no saturation occurred. Moreover, no significant differences (α = 0.05) were observed between inter-day excreted relative amounts. As shown in Figure 1, 19-norandrosterone and 19-noretiocholanolone are the major metabolites in the chimeric mice. This is in agreement with previous studies in humans.[8–11] Indeed, these 2 metabolites are only detectable in the chimeric mouse urine but not in the non-chimeric mouse urine. Hence, their presence can be assigned to the human hepatocytes in the chimeric mouse liver and therefore confirms that these metabolites are specific markers of 19-norAD metabolization by human hepatocytes.

These results also indicate that transplantation of the murine liver results in a shift of metabolism towards a human metabolic

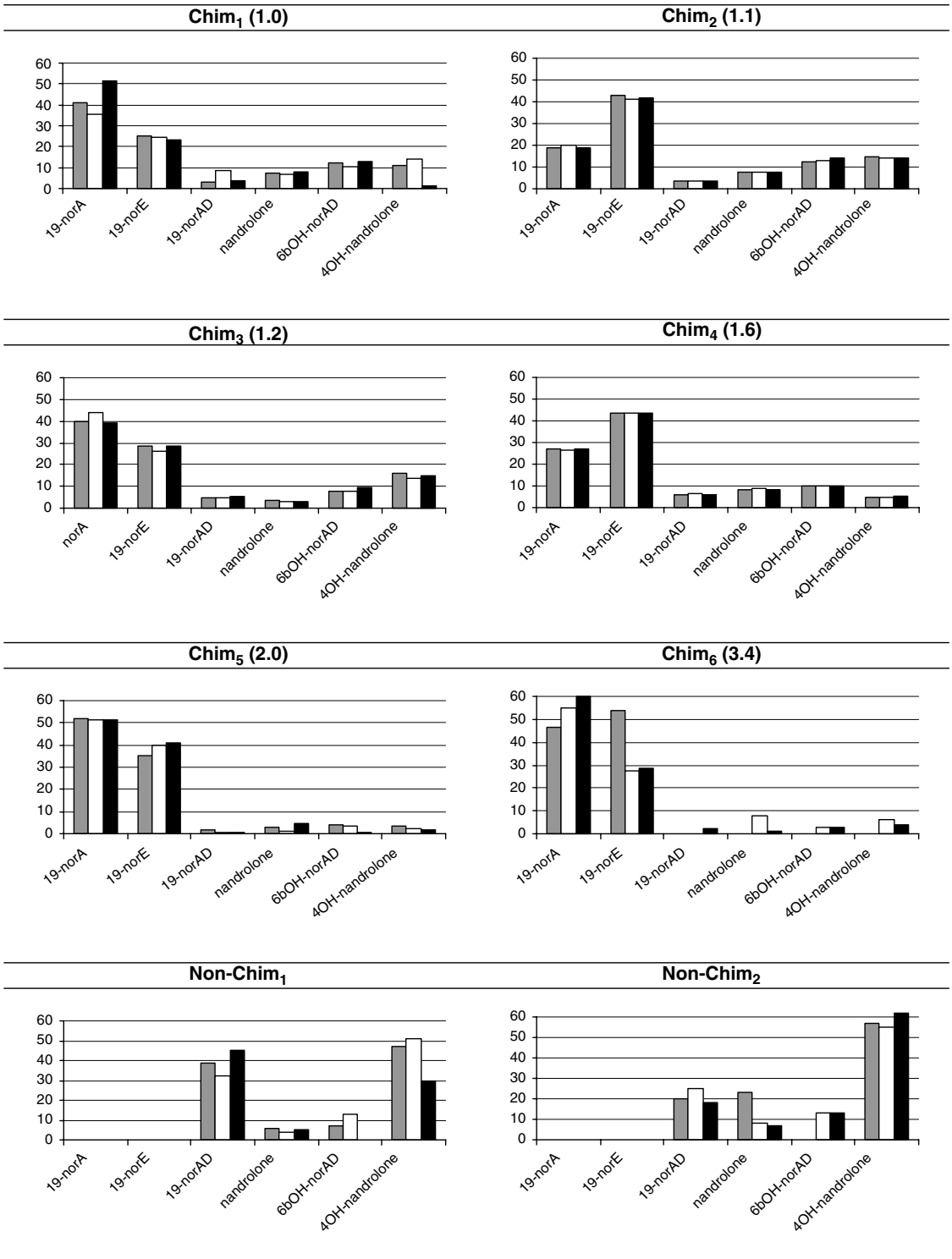


Figure 1. The metabolism was studied following the three-day administration (Day 1-2-3) of a 19-norAD-PBS suspension in six chimeric mice (Chim1-6) and two non-chimeric mice (Non-Chim1-2). The relative abundance of 19-norAD and its metabolites is presented. The human albumin concentration (mg/mL) of the chimeric mice is indicated between brackets.
Legend:
19-norandrostenedione – 19-norA
19-noretiocholanolone – 19-norE
19-norandrost-4-ene-3,17-dione – 19-norAD
nandrolone, 6β-hydroxynorandrostenedione – 6bOH-norAD
4-hydroxynandrolone – 4OH-nandrolone
Day 1: gray, Day 2: white, Day 3: black.

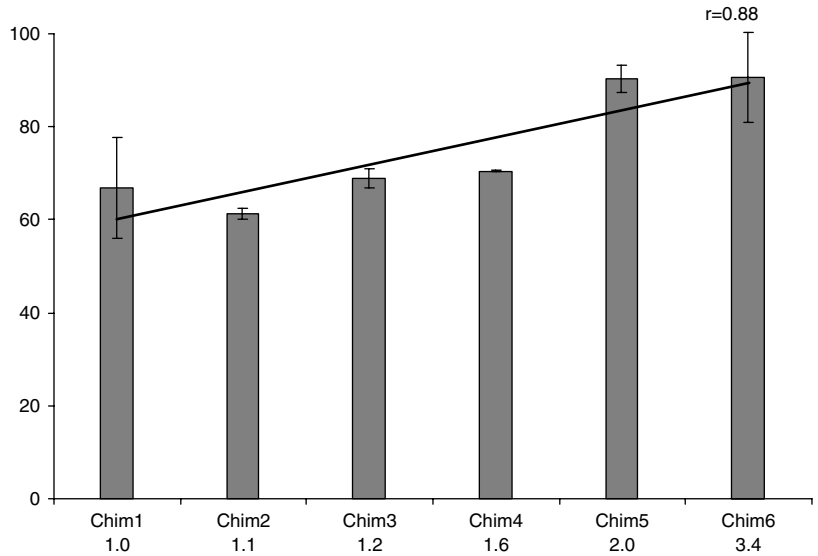


Figure 2. Overview of the sum of the mean relative abundance (n = 3) of 19-norandrosterone and 19-noretiocholanolone, the major metabolites after 19-norAD administration to six chimeric mice. The chimeric mice are ranked according to their increasing plasma human albumin (hAlb, mg/mL) concentration. Two human hepatocytes donors are used: Chim1-4: type a, Chim5-6: type b.

profile. This is further highlighted by the fact that higher plasma hAlb concentrations lead to increasing relative amounts of 19-norandrosterone and 19-noretiocholanolone and to decreasing levels of the other metabolites that are also present in the urine of non-chimeric mice (Figure 1). 6β-hydroxynorandrostenedione was detected as a minor metabolite in the chimeric mouse, which confirms previous findings by Schänzer in humans.[10]

Analysis of the chimeric mouse post-administration urine revealed the presence of three additional metabolites. Based upon published mass spectra,[10] it can be assumed that these peaks correspond to 16α-hydroxynorandrosterone, 16α-hydroxynoretiocholanolone and 16α-hydroxynorepiandrosterone. Unfortunately no reference standard was available to unequivocally confirm their identity.

To exclude the possibility that the results might be influenced by specific biological characteristics of the hepatocyte donor, six chimeric mice transplanted with two types of human hepatocytes donors were used. No difference in the detection of the monitored compounds could be observed between both types of chimeric mice (type a: Chim1-4; type b: Chim5-6). Only a significant variability in the distribution of the relative abundance of the metabolites between the chimeric mice was noticed (Figure 1 and 2). In humans, 19-norandrosterone is considered to be the major metabolite after 19-nor-ρ 4 steroid administration, whereas predominance of 19-noretiocholanolone over 19-norandrosterone is an indication of 19-nor-ρ 5 steroid administration.[8] In contrast to humans, such a correlation was not observed in the chimeric mice (Figure 1).

The chimeric mice have increasing plasma hAlb concentrations. The hAlb is measured as a marker for successful transplantation of functional human hepatocytes. Indeed, previously Tateno *et al*.[17] showed a positive correlation between the extent of replacement of the liver with human hepatocytes and the hAlb concentration.

In the 19-norAD study, a positive correlation (r = 0.88) was found between the plasma hAlb concentration of the chimeric mice and the sum of the relative amounts of 19-norandrosterone and 19-noretiocholanolone (Figure 2). This confirms that the relative abundance of the major human metabolites increases in the chimeric mice in a hAlb concentration-dependent manner.

As shown in Figure 2, 19-norandrosterone and 19-noretiocholanolone represent a higher amount of the metabolites in the chimeric mice of donor type b. This can be assigned to the high hAlb concentrations, indicating a high amount of human hepatocytes present in the mouse liver, rather than the donor type. Based upon these experiments with 19-norAD, it can be concluded that the influence of the characteristics of the cell donor is quite limited, as long as the hAlb concentration in murine plasma is high (Figure 2). Nevertheless further research is necessary to confirm these findings.

These results indicate that the successful transplantation with human hepatocytes determines the steroid metabolism in the mice and that based upon the observed differences between chimeric and non-chimeric mice, extrapolation of data towards metabolism in humans becomes possible.

Evaluation of the chimeric mouse model

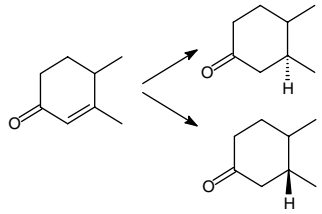
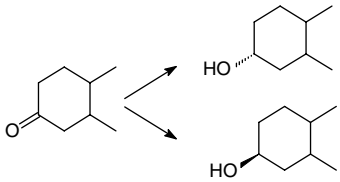
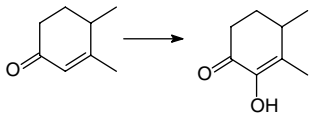
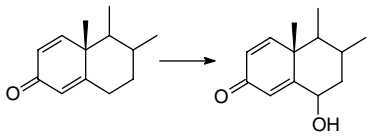
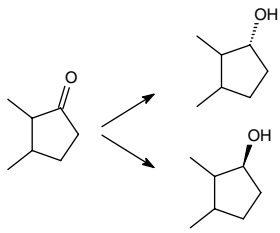
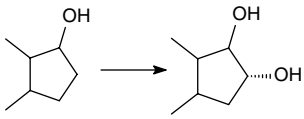
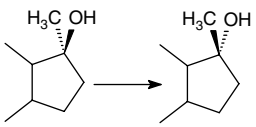
Metabolic pathways

The metabolism in the chimeric mouse model was validated with 19-norAD and two other selected steroids. The results for AD[13] and MTD[14,18] have been described previously. An overview of the detected phase I steroid metabolic pathways in the chimeric mice is shown in Table 2.

3-Keto-reduction and 5α/β–reduction was observed for the three administered steroids in the chimeric mouse. These pathways are amongst the most important metabolic pathways for most steroids and were in general missing in the non-chimeric mice.[19] 6-Hydroxylation has been described in humans[19] and this pathway has now been confirmed using the chimeric mouse model. Moreover the stereospecificity of 6-hydroxylation was maintained in the mouse model for methandienone[14] (β), androstenedione[13] (α) and 19-norAD (β) (Table 2).

As in humans, 4-hydroxylation of AD[20] and 19-norAD was observed in chimeric mice, but not of MTD. Metabolic alterations of the D-ring were similar to those in humans, namely 17-keto

Table 2. A general overview of the phase I metabolic pathways detected in chimeric mice based on the administration studies with androst-4-ene-3,17-dione (AD), methandienone (MTD) and 19-norandrost-4-ene-3,17-dione (19-norAD)

Phase I metabolism			
A-ring	5α- and 5β- reduction	AD MTD 19-norAD	
	3-keto-reduction	AD MTD 19-norAD	
	4-hydroxylation	AD 19-norAD	
B-ring	6-hydroxylation	AD (α) MTD (β) 19-norAD (β)	
D-ring	17-keto-reduction	AD 19-norAD	
	16α-hydroxylation	AD	
	17-epimerization	MTD	

reduction for AD and 19-norAD, 16α-hydroxylation for AD and 17-epimerization for MTD. This epimerization pathway occurs via a sulphated intermediate and hence provides indirect evidence of phase II enzymatic pathways in the chimeric mouse.

In addition to phase I metabolic reactions, phase II glucuronidation or sulphatation of the steroids was also checked in a preliminary study. Phase II metabolism in the chimeric mice seems to correspond to phase II metabolism in humans. It should be noted that in humans the major metabolites of the investigated steroids are mainly excreted as free or glucuronide and less as sulphates.[14] Moreover, hydrolysis of sulphates results in much dirtier extracts. The urine samples of the effective administration study were extracted after *E. coli* hydrolysis to obtain the combined fraction of free metabolites and glucuronides.

In summary, the results of the validation of this model showed large differences between the chimeric (with humanized liver) and non-chimeric mice and indicated that the most prominent metabolic pathways in humans are present in the chimeric mouse model. The uPA$^{+/+}$-SCID mouse model with humanized liver has proven to be a good and appropriate model to mimic human steroid metabolism for those steroids for which it has been validated (19-norAD, AD and MTD).

Practical issues

Although the chimeric mouse is a promising model with respect to the array of metabolic pathways and the excellent correlation with the metabolism in humans, some limitations are inherent such as the relatively low amount of urine that can be collected and the concentrated mouse urine matrix. Due to the low urine production in mice, it should be noted that the amount of mouse urine taken for analysis in the presented studies was only 100–500 μL.

Moreover, mouse urine is a very concentrated matrix, which results in higher LOD because of increased background[13] or higher ion suppression.[14] Therefore higher administration doses are needed. To optimize the 19-norAD administration dose, an amount 10 times higher per kg/bodyweight compared to humans was administered. Nevertheless, administration can be performed repeatedly in relatively high doses over a longer period of time, which is an advantage over *in vitro* cultures.[5]

These practical issues, however, do not outweigh the advantages of the chimeric mouse as an *in vivo* model to generate human-type metabolites. Moreover, due to their relatively long lifespan, the chimeric mice can be reused for multiple administrations.

Conclusion

The aim of this study was to demonstrate the usefulness of chimeric mice as an *in vivo* model for the identification of human metabolites. The model was validated by comparison of results from chimeric and non-chimeric mice with previously reported data for three model steroids (AD, MTD, 19-norAD) in humans. These steroids were selected to assess the *in vivo* steroid metabolism potential of chimeric mice, because a comprehensive set of data on the metabolism of these steroids in humans and *in vitro* studies was available for comparison.

After 19-norAD administration, it was possible to indentify, by GC-MS, several human metabolites formed in chimeric and non-chimeric mice. However, the major human metabolites 19-norandrosterone and 19-noretiocholanolone were exclusively present in the urine of the chimeric mice and were the major metabolites as well.

Based on the results of the 19-norAD, MTD and AD administration studies it can be concluded that the chimeric mice transplanted with primary human hepatocytes mimic the human metabolism. Moreover, increased hepatocyte replacement leads to a better correlation with the human *in vivo* metabolism.

The results of the studies with the three selected steroids show the potential use of the chimeric mouse as a suitable (complementary) model to other *in vivo* animal models or *in vitro* cultures. The presence of a wide range of metabolic pathways has been tested and it has been proven that the chimeric mouse model can produce human-type metabolites. The results suggest that this *in vivo* animal model has good discriminatory potential to detect the most suitable steroid metabolites for screening purposes in doping control. From these results it is believed that the chimeric mice could contribute to the discovery and identification of new metabolites of banned doping agents by acting as a predictive model.

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