uPA⁺/⁺-SCID Mouse with Humanized Liver as a Model for In Vivo Metabolism of Exogenous Steroids: Methandienone as a Case Study

Leen Lootens,1* Philip Meuleman,2 Oscar J. Pozo,1 Peter Van Eenoo,1 Geert Leroux-Roels,2 and Frans T. Delbeke1

BACKGROUND: Adequate detection of designer steroids in the urine of athletes is still a challenge in doping control analysis and requires knowledge of steroid metabolism. In this study we investigated whether uPA⁺/⁺-SCID mice carrying functional primary human hepatocytes in their liver would provide a suitable alternative small animal model for the investigation of human steroid metabolism in vivo.

METHODS: A quantitative method based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) was developed and validated for the urinary detection of 7 known methandienone metabolites. Application of this method to urine samples from humanized mice after methandienone administration allowed for comparison with data from in vivo human samples and with reported methandienone data from in vitro hepatocyte cultures.

RESULTS: The LC-MS/MS method validation in mouse and human urine indicated good linearity, precision, and recovery. Using this method we quantified 6 of 7 known human methandienone metabolites in the urine of chimeric mice, whereas in control nonchimeric mice we detected only 2 metabolites. These results correlated very well with methandienone metabolism in humans. In addition, we detected 4 isomers of methandienone metabolites in both human and chimeric mouse urine. One of these isomers has never been reported before.

CONCLUSIONS: The results of this proof-of-concept study indicate that the human liver–uPA⁺/⁺-SCID mouse appears to be a suitable small animal model for the investigation of human-type metabolism of anabolic steroids and possibly also for other types of drugs and medications.

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The World Anti-Doping Agency has forbidden the use of anabolic androgenic steroids because of their performance-enhancing effects in sports (1). Recently a number of designer anabolic steroids have been introduced on the nutritional supplement market and are sold over the internet without information about toxicological effects or their metabolism. For ethical and safety reasons, it is difficult to perform in vivo human excretion studies to investigate the metabolism of designer steroids.

Liver fractions (2), hepatocyte cell cultures (3–5), and primates (6, 7) have been used as alternatives to human administration in studies of metabolism. These alternative models are not ideal, however. Human hepatoma cells have several metabolic defects; cultured primary hepatocytes lose their differentiation early after culture, which affects their metabolic activity; and nonhuman primate studies are subject to the same ethical constraints that prevent human studies (8–10). Thus the development of efficient detection methods to identify metabolites of new performance-enhancing substances in urine is severely hampered. Therefore alternative metabolism models need to be developed.

In this study the chimeric uPA⁺/⁺-SCID mouse (11–13) was evaluated as a possible alternative model for use in determining the human-type metabolism of steroids in vivo. uPA⁺/⁺-SCID mice are immunodeficient mice suffering from a chronic liver disease caused by the liver-specific overexpression of the uPA gene, plasminogen activator, urokinase (PLAU) (14). The liver disease in these mice can be cured by transplantation of primary human hepatocytes. These human hepatocytes migrate to and repopulate the liver and restore normal liver function (11, 12). Human liver cell expansion and function in these mice can be evaluated a few weeks after transplantation by measurement of the human albumin concentration in the mouse plasma, which serves as a reliable in vivo marker.
Optimal repopulation requires transplantation of high-quality human hepatocytes within a short period after birth.

The chimeric mouse model has previously been used for a wide range of applications, and P450 enzymes, such as CYP3A4, CYP1A1, and CYP1A2, can easily be induced in chimeric mice (15). With the use of cefmetazole as a probe drug, the excretory profile of chimeric mice was shown to be similar to that of humans (16), a finding that suggests that chimeric mice may be a useful animal model in excretion studies.

Chimeric mice may contribute to the elucidation of the metabolism of new designer steroids. Theoretically, this in vivo mouse model could offer a perfect alternative to the suboptimal method of using in vitro cultures. Before application, the chimeric mouse model must be validated using previously studied steroids. For doping control purposes it is important to know which metabolites are formed, to allow for the detection of prohibited substances in urine. Because of the first-pass effect in the liver, oral administration of 17-methyltestosterone is not really effective unless structural changes (e.g., alkyl group at C17) are introduced to cir-

Chimeric mice. The structures of the selected analytes are shown in Fig. 1. 17α-Methyltestosterone (I); methandienone (II); 17-epimethandienone (III); 17β-methyl-5β-androsta-1,3α,17α-diol (V; epimethandienol); 17,17-dimethyl-18-norandrost-1,4,13-trien-3-one (VI); 17,17-dimethyl-18-nor-5β-androsta-1,3-dien-3α-ol (IX) were purchased from the National Measurement Institute. 17α-Methyl-5β-androst-1-en-17β-ol-3-one (IV) was bought from Steraloids. 17β-Hydroxyethyl-17α-methyl-18 norandrost-1,4,13-trien-3-one (VII); 17α-hydroxyethyl-17α-methyl-18-norandrost-1,4,13 trien-3-one (epimer of VII=VIIc); and 17α-methyl 5β-androsta-3α,17β-diol (VIII) were a gift from the Institut für Biochemie of the Deutsche Sporthochschule in Cologne, Germany. The internal standard 17α-methyltestosterone was a gift from Organon.

HPLC grade methanol and water were from Acros and Fischer, respectively. Ammonium acetate was from Sigma. Diethyl ether was obtained from Biosolve. Na2SO4, NaHCO3, and K2CO3 were purchased from Merck. β-Glucuronidase from Escherichia coli K12 was from Roche Diagnostics. For the hydrolysis of sulfates, β-glucuronidase from Helix pomatia was obtained from Sigma-Aldrich.

**Materials and Methods**

**CHEMICALS AND REAGENTS**

The structures of the selected analytes are shown in Fig. 1. 17α-Methyltestosterone (I); methandienone (II); 17-epimethandienone (III); 17β-methyl-5β-androsta-1,3α,17α-diol (V; epimethandienol); 17,17-dimethyl-18-norandrost-1,4,13-trien-3-one (VI); 17,17-dimethyl-18-nor-5β-androsta-1,3-dien-3α-ol (IX) were purchased from the National Measurement Institute. 17α-Methyl-5β-androst-1-en-17β-ol-3-one (IV) was bought from Steraloids. 17β-Hydroxyethyl-17α-methyl-18-norandrost-1,4,13-trien-3-one (VII); 17α-hydroxyethyl-17α-methyl-18-norandrost-1,4,13-trien-3-one (epimer of VII=VIIc); and 17α-methyl-5β-androsta-3α,17β-diol (VIII) were a gift from the}

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**PRODUCTION AND DOSING OF uPA-SCID MICE**

Chimeric mice were produced as described previously (11). Briefly, uPA+/-SCID mice received transplants within 2 weeks after birth with primary human hepatocytes (chimeric mice) (13). uPA-SCID mice that did not receive transplants with human hepatocytes served as a control group (nonchimeric mice). The project was approved by the Animal Ethics Committee of the Faculty of Medicine of Ghent University (ECD 06/09).

To chimeric and nonchimeric mice, 100 μL of a PBS suspension of methandienone (400 mg/L) was administered via oral gavage. Applying a double-blind study protocol, we gave mice a placebo as an extra control parameter. The mouse urine was collected separately from the feces by use of special metabolic cages designed for small rodents (Tecniplast). Blank urine samples were collected 24 h before dosage. Afterward, urine was collected every 24 h and stored at −20 °C until analysis. The mice had ad libitum access to water and powdered food. The blank mouse urine, used to prepare the calibration curve and positive and negative control samples, was tested before use.

**SAMPLE PREPARATION**

For the analysis of the urine samples, an internal standard solution (25 μL of 17α-methyltestosterone, 2 mg/L) was evaporated under oxygen-free nitrogen in a 10-mL glass tube, and then 500 μL of urine was added. The urine was hydrolyzed (2.5 h at 56 °C) after addition of 1 mL phosphate buffer (pH 7) and 50 μL of E. coli β-glucuronidase solution [for hydrolysis of sulfates: 1 mL acetic buffer (pH 5.2) and 50 μL of β-glucuronidase from Helix pomatia were used]. After cooling to room temperature, 200 mg of solid buffer NaHCO3/K2CO3 (2:1) was added. A liquid-liquid extraction was performed with 5 mL diethyl ether by rolling for 20 min, followed by 5-min centrifugation at 1200g. The organic layer was separated and evaporated under nitrogen at 40 °C. The residue was dissolved into 100 μL of H2O:methanol (1:1).
An HPLC Finnigan Surveyor MS pump plus (Thermo) was interfaced to a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo) using the electrospray interface; 20 μL of the sample was injected into the system using a Finnigan surveyor autosampler plus (Thermo). The chromatographic separation was performed on a Varian Omnispher C18 column (i.d. 100 × 2 mm, 3 μm) (Varian) maintained at 35 °C, at a flow rate of 250 μL/min, using a ChromSep guard column (i.d. 10 × 2 mm, 5 μm) (Varian). Mobile-phase solvents were 1 mmol/L ammonium acetate in water (A) and 1 mmol/L ammonium acetate in methanol (B). A gradient program was used, and the percentage of organic solvent (B) was changed.

Fig. 1. Overview of methandienone metabolism.
I, methandienone; II, 6β-hydroxymethandienone; III, 17-epimethandienone; IV, 17α-methyl-5β-androsta-1-en-17β-ol-3-one; V, epimethadiol; VI, 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one; VII, 17β-hydroxymethyl-17βα-methyl-18-norandrosta-1,4,13-trien-3-one; VIII, 17α-methyl-5β-androsta-3α,17β-diol; IX, 17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol.
linearily as follows: 0 min, 25%; 1.5 min, 25%; 15 min, 95%; 18 min, 95%; 19 min, 25%; 22 min, 25%.

Nitrogen was used as sheath gas, ion-sweep gas, and auxiliary gas, at flows of 50, 2, and 20 U, respectively. A spray voltage of 4000 V was used in positive ionization mode. The capillary temperature was set to 350 °C, and the source collision–induced dissociation to 2 U. The collision gas was argon (Air Liquide, Destel-donk) with a collision gas pressure of 1.5 mTorr.

EXTRACTION EFFICIENCY AND ION SUPPRESSION

For extraction efficiency experiments, 6 blank mouse and human urine samples were spiked with 50 μg/L of all analytes, and extraction was performed as described above. In addition 6 blank urine samples were extracted, and afterward the transferred organic layer was spiked at 50 μg/L, simulating a 100% extraction efficiency. Both sets of extracts were then analyzed with the described liquid chromatography–tandem mass spectrometry (LC-MS/MS) method. The extraction efficiency was calculated by comparison of the area obtained for the samples spiked before and after extraction.

For evaluating ion suppression, 6 different blank urine samples were extracted and then spiked at 50 μg/L with all analytes to avoid losses during the extraction procedure. The ion suppression was calculated by comparing the responses between these spiked extracts and a standard at the same concentration prepared in mobile phase. The SD of the ion suppressions was calculated to evaluate the reproducibility, i.e., the variation between different urine matrices.

METHOD VALIDATION

The validation of the method in mouse and human urine was performed according to the Eurachem guidelines for quantitative methods (29). For linearity, 7–point calibration curves (between 5 μg/L and 500 μg/L) were prepared after spiking blank urine of the respective species. Because of the small amount available of the reference standard, only a 5–point calibration curve for metabolite VII was prepared. Each of the concentration levels was analyzed 6 times and was prepared in different urine matrices. The calibration curves were calculated by use of a least squares fit analysis.

The percentage recovery of the method was expressed as: (measured value/ assigned value) × 100. Imprecision was evaluated by determination of the repeatability (n = 6, same analyst) at various concentrations. Repeatability was calculated as the relative standard deviation (CV). Maximum allowed values (%) for repeatability can be calculated from the Horwitz equation via the maximum relative SD: CV max = 2(1 – 0.5logC) [C = concentration (μg/mL) × 10^-6] (30).

The limit of quantification (LOQ) of the method was determined as the lowest concentration for which a compound can be detected with acceptable precision (measured by being less than two-thirds of the Horwitz maximum repeatability CV, Table 2). The limit of detection was set arbitrarily at one-half of the LOQ. The specificity of the method was tested by analyzing other doping agents.

Results and Discussion

LC-MS/MS METHOD DEVELOPMENT

The LC-MS/MS method was developed for the quantitative measurement of methandienone and its metabolites in 500 μL of mouse and human urine. The retention times of the monitored substances are presented in Table 1.

Phase II metabolism was also tested during method development. Therefore urine from the chimeric mouse was first extracted free, followed by an extraction after hydrolysis with β-glucuronidase from E. coli and finally extraction after hydrolysis with β-glucuronidase from H. pomatia for the sulfated fraction. Methandienone metabolites II, III, and VI were excreted unconjugated (free), whereas metabolite IV and methandienone itself (I) were mainly excreted glucuronidated. These chimeric mouse results correlate with previously reported data of phase II metabolism in humans (20, 24). After enzymatic hydrolysis with β-glucuronidase from H. pomatia, no noteworthy concentration (5% or higher) was measured in the sulfated fractions for any of the excreted metabolites. Therefore the sulfated fractions were not studied further, and only an E. coli glucuronidase hydrolysis was incorporated in the analytical procedure; hence both free and glucuronidated metabolites were detected.

Most analytes have a keto group at C3 position and therefore could be monitored as [M+H]^+ (31). Epimethandiol (V), without keto function in C3, was detected as [M+NH4+H2O]^+ in the positive ionization mode (Table 1). The main product ions observed are shown in Table 1. The method was able to quantify all commercially available methandienone metabolites in mouse urine except 17α-methyl-5β-androsta-3α, 17β-diol (VIII), and 17β,17-dimethyl-18-nor-5β-androsta-1,3-dien-3α-ol (IX) (Fig. 1). Owing to ionization problems in LC-MS using electrospray (31), these compounds could not be detected at sufficiently low concentrations.
For LC optimization a good separation was needed, because several compounds show the same precursor ion, like m/z 301 for I and III and m/z 303 for IV and the internal standard (Table 1; also see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue10). Additionally, some of the ions were generated by insource fragmentation after losses of water [M+H-H2O]+, generating the same mass as other compounds. As an example, monitoring the transition for metabolite VII (299→147) also resulted in a signal for metabolite II ([M+H]+ 317). After insource fragmentation of metabolite II (m/z 317), the transition 299→147 was also produced (online Supplemental Fig. 1). A similar situation was observed for metabolite VI, with which I and III could also be monitored (online Supplemental Fig. 1).

**EXTRACTION EFFICIENCY AND ION SUPPRESSION**

The ion suppression for mouse and human urine was calculated for all compounds (Table 1). In general, the ion suppression is much higher for the early-eluting polar compounds like 6β-hydroxymethandienone (II), and lower for the later-eluting less polar compounds. Ion suppression was also higher in mouse urine than in human urine.

Extraction efficiencies for the steroids in mouse and human urine were given in Table 2. The correlation coefficients (r²) were at least 0.98 for all analytes. For each compound the LOQ was the lowest or the second point of the calibration curve (Table 2) except for epimethendiol (V), for which the LOQ was higher (25 μg/L). A chromatogram of a blank and spiked mouse urine sample is presented in online Supplemental Fig. 1.

The imprecision of the method was tested at selected concentrations (Table 2) for each compound. From the results in Table 2 it is clear that the repeatability imprecision never exceeded the limits set by two-thirds of the CVmax and all values were also within the limits of ±20% for the bias. Hence the method was precise over the linear range of the calibration curve.

For the validation of the LC-MS/MS method in human urine, similar results for linearity, bias and precision were obtained (data not shown).

The described method is selective because no interferences were detected by analyzing reference standards of other doping agents for which screening is routinely performed, including 16 narcotics, 11 corticosteroids, 27 exogenous anabolic androgenic steroids, 7 β-agonists, 21 β-blockers, 28 diuretics, and 54 stimulants.

**ANALYSIS OF POSTADMINISTRATION METHANDIENONE SAMPLES**

**HUMAN URINE SAMPLES**

Once the method was validated, 20 samples positive for methandienone from earlier excretion studies with human participants and proficiency testing samples from

### Table 1. Retention times, monitored m/z values in the positive ionization mode, instrumental settings, and extraction efficiency and ion suppression with relative SD in mouse and human urine.

<table>
<thead>
<tr>
<th>RT, min</th>
<th>Metabolite</th>
<th>Precursor ion</th>
<th>Production, m/z</th>
<th>Collision energy, eV</th>
<th>Extraction efficiency (50 μg/L), mean ± RSD, % (n = 6)</th>
<th>Ion suppression, mean ± RSD, % (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.3</td>
<td>II</td>
<td>[M+H]+</td>
<td>317</td>
<td>171</td>
<td>88 ± 20/82 ± 5 (Human/Mouse) -47 ± 2/-80 ± 13 (Human/Mouse)</td>
<td></td>
</tr>
<tr>
<td>11.9</td>
<td>I</td>
<td>[M+H]+</td>
<td>301</td>
<td>121</td>
<td>92 ± 15/84 ± 6 (Human/Mouse) -48 ± 2/-64 ± 10 (Human/Mouse)</td>
<td></td>
</tr>
<tr>
<td>13.1</td>
<td>III</td>
<td>[M+H]+</td>
<td>301</td>
<td>121</td>
<td>92 ± 8/83 ± 8 (Human/Mouse) -5 ± 3/-14 ± 10 (Human/Mouse)</td>
<td></td>
</tr>
<tr>
<td>13.0</td>
<td>VII</td>
<td>[M+H]+</td>
<td>299</td>
<td>147</td>
<td>88 ± 8/96 ± 8 (Human/Mouse) -2 ± 5/-22 ± 11 (Human/Mouse)</td>
<td></td>
</tr>
<tr>
<td>13.4</td>
<td>IV</td>
<td>[M+H]+</td>
<td>303</td>
<td>201</td>
<td>97 ± 17/87 ± 7 (Human/Mouse) -22 ± 4/-44 ± 16 (Human/Mouse)</td>
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<tr>
<td>12.7</td>
<td>ISTD</td>
<td>[M+H]+</td>
<td>303</td>
<td>97</td>
<td>—/— (Human/Mouse) —/— (Human/Mouse)</td>
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<tr>
<td>16.1</td>
<td>VI</td>
<td>[M+H]+</td>
<td>283</td>
<td>147</td>
<td>86 ± 6/73 ± 12 (Human/Mouse) -22 ± 12/-27 ± 19 (Human/Mouse)</td>
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<tr>
<td>16.3</td>
<td>V</td>
<td>[M+NH4H2O]+</td>
<td>304</td>
<td>269</td>
<td>79 ± 13/75 ± 14 (Human/Mouse) -9 ± 4/-20 ± 13 (Human/Mouse)</td>
<td></td>
</tr>
</tbody>
</table>

*RT, retention time; RSD, relative SD.

Metabolites: I, methandienone; II, 6β-hydroxymethandienone; III, 17-epimethandienone; IV, 17α-methyl-5β-androsta-1-en-17β-ol-3-one; V, epimethendiol; VI, 17,17-dimethyl-18-norandrosta-1,4,13-tien-3-one; VII, 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-tien-3-one; ISTD, internal standard, 17α-methyltestosterone.
the World Anti-Doping Agency were analyzed. In these untimed human urine samples, the concentrations found varied highly from sample to sample. To make the data more easily understood, the results have been presented in box-and-whisker plots (Fig. 2). The metabolite 6β-hydroxymethandienone (II), detected in all samples, was set as 100%. In humans, 6 commercially available metabolites and methandienone itself could be quantified. However, in some samples [as shown in Fig. 2 methandienone (I) or metabolites IV, V, VI or VII] could not be detected. Only metabolites II and III could be quantified in all human urine samples.

**MOUSE URINE SAMPLES**

Pilot studies to detect any saturation problem were performed by administering 100 μL of increasing concentrations of methandienone (100, 200, or 400 mg/L). When we analyzed the samples we observed no saturation effects, so the highest dose was selected for the study, because it gave the most abundant signals for the metabolites.

To achieve sufficiently high concentrations for detection, the orally administered methandienone dose (40 μg) given to the mice (± 15 g) was 20 times higher per kilogram body weight than the recommended therapeutic dose in humans. The mice produce an average of only 1.5 mL of urine a day. The mouse urine collected on the day before administration or from the mice receiving the placebo solution was blank. Application of the LC-MS/MS method to the mouse urine samples collected 24 h after methandienone administration resulted in substantial differences between chimeric and nonchimeric mice (Fig. 2). In the nonchimeric mouse urine only the parent compound methandienone (I) and its main metabolite 6β-hydroxymethandienone (II) could be detected (Figs. 2

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### Table 2. LC-MS/MS validation parameters for the quantitative measurement of methandienone and metabolites in mouse urine.

<table>
<thead>
<tr>
<th>Calibration values, μg/L</th>
<th>Metabolite</th>
<th>Correlation coefficient ($r^2$)</th>
<th>LOQ, μg/L</th>
<th>Concentration, μg/L</th>
<th>CV, % (n = 6)</th>
<th>$&lt;\frac{2}{3}$ RSD$_{max}$ b</th>
<th>Recovery, % (n = 6)</th>
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</thead>
<tbody>
<tr>
<td>5, 10, 25, 50, 100</td>
<td>VII</td>
<td>0.99</td>
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<td>10</td>
<td>4</td>
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<tr>
<td>5, 10, 25, 50, 100, 200, 500</td>
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<td>21.3</td>
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<td>200</td>
<td>9</td>
<td>13.6</td>
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</tr>
</tbody>
</table>

*a Metabolites: I, methandienone; II, 6β-hydroxymethandienone; III, 17-epimethandienone; IV, 17α-methyl-5β-androsta-1-en-17β-ol-3-one; V, epimethendiol; VI, 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one; VII, 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one; RSD, relative SD.

*b At selected concentrations the repeatability was measured. Maximum allowed values for repeatability can be calculated from the relative SD (RSD)$_{max}$ Horwitz equation (30). The repeatability must be lower than 2/3 of the calculated RSD$_{max}$.
and 3). On the other hand, 6 methandienone metabolites were present in the chimeric mouse urine, indicating the contribution of the human hepatocytes in the chimeric mouse model. Only epimethendiol (V) could not be detected in the chimeric mouse urine (Fig. 2).

**COMPARISON BETWEEN MOUSE AND HUMAN RESULTS**

The results from human administration studies were compared with those of the chimeric and nonchimeric mouse urine after administration of methandienone. When we compared the chimeric mouse urine with human urine, the results showed a correlation of at least 85%, because 6 of the 7 methandienone metabolites could be quantified in the chimeric mouse urine. Only epimethendiol (V) could not be detected. In humans also, however, substantial variation seems to occur in epimethendiol concentrations (Fig. 2). In 6 of the 20 human postadministration samples, no epimethendiol could be detected either. In view of the low concentration or even absence of epimethendiol in urine of some humans and the low volume of urine used in this method combined with the relatively high limit of detection for this substance (Table 2), we cannot exclude the possibility that epimethendiol is a minor metabolite (undetectable under the current circumstances) in the chimeric mouse.

When comparing the relative abundances of methandienone and its metabolites in chimeric mice with those in humans, the correlation between both is significant (Figs. 2 and 3). Moreover the metabolites were detected in similar relative abundances in mice as in humans. The results show that urine of chimeric mice contains multiple metabolites that were not previously reported in any in vitro culture (2, 28). In addition to 6β-hydroxylation, multiple other metabolic pathways were found in the chimeric mouse, including 17-epimerization, 5β-reduction, and 3α-dehydrogenation. These data show the direct applicability of this method for the assessment of the presence of methandienone metabolites in the urine of mice.

**Fig. 2.** Box-and-whisker plot indicating the distribution for methandienone and its metabolites. The untimed methandienone concentrations are set relative to 6β-hydroxymethandienone (II) = 100% in (a) 20 positive excretion urine samples of humans and (b) the relative amounts of nonchimeric and (c) chimeric mouse urine after methandienone administration. Missing plot symbols for metabolites in the chimeric or nonchimeric urines indicate that the metabolite was undetectable. I, methandienone; II, 6β-hydroxymethandienone; III, 17-epimethandienone; IV, 17α-methyl-5β-androsta-1-en-17β-ol-3-one; V, epimethendiol; VI, 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one; VII, 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one; VIII, 17α-methyl-5β-androsta-3α,17β-diol; IX, 17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol; ISTD, internal standard.
Fig. 3. LC-MS/MS chromatograms for methandienone metabolites detected in (a) human, (b) chimeric mouse, and (c) nonchimeric mouse urine samples.

The x-axis for each chromatogram (representing the chromatography time) is scaled identically for each metabolite, and the y-axis gives relative abundances. Detailed information about the observed retention times of peaks can be obtained by enlarging the online electronic version of this Fig in the online version of this article at http://www.clinchem.org/content/vol55/issue10. For transitions see Table 1. I, methandienone; II, 6β-hydroxymethandienone; III, 17-epimethandienone; IV, 17α-methyl-5β-androsta-1-en-17β-ol-3-one; V, epimethendiol; VI, 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one; VII, 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one; VIII, 17α-methyl-5β-androsta-3α,17β-diol; IX, 17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol.
with humanized liver and indicate that similar metabolic pathways as in humans could be observed in the chimeric mouse. The chimeric mouse LC chromatograms (Fig. 3) are almost a perfect copy of those obtained with human urine.

ADDITIONAL DETECTED ISOMERS

Although the method was developed for the quantification of 7 compounds for which reference standards were available, additional metabolites were detected owing to the optimized LC gradient. Indeed, with this method 11 compounds could be detected in total. The 4 additional compounds, detected after analysis of the chimeric mouse urine, were isomers of metabolites VI and VII (Fig. 3). Importantly, their presence was confirmed in the human urine samples as well.

17,17-DIMETHYL-18-NORANDROSTA-1,4,13-TRIEN-3-ONE (METABOLITE VI)

In the urine of humans and chimeric mice 1 isomer of VI was detected. The LC-MS/MS chromatograms illustrate the good correlation between humans (Fig. 3a) and the chimeric mice (Fig. 3b), and the difference with the nonchimeric control mice (Fig. 3c), in which neither VI nor the isomer (VIa) could be detected.

Metabolite VI and its isomer are byproducts of a 17-epimerization reaction (Fig. 1). It has previously been reported that epimerization can lead to different metabolites through an intermediate sulfate conjugate (21, 22, 24, 25, 32, 33). The 17β-sulfate hydrolyzes in water to several products, including metabolites III and VI. The isomer of VI, found with this LC-MS/MS method, was detected in the chimeric mouse urine and in 15 of the 20 human urine samples. When the isomer was detectable, the isomer intensity was around 10%–20% that of metabolite VI. Further investigation is necessary to elucidate the structure of this VI-epimer.

17β-HYDROXYMETHYL-17α-METHYL-18-NORANDROSTA-1,4,13-TRIEN-3-ONE (METABOLITE VII)

The discovery of VII as a long-term metabolite of methandienone (Fig. 1) led to a significant improvement of detection times for methandienone use (17).

Analysis of the chimeric mouse urine after methandienone administration resulted in the detection of 3 peaks (VIIa), (VII), and (VIIb) for m/z 299 (Fig. 3). Analysis of the excretion in human urine samples confirmed the presence of peak (VIIa), (VII), and (VIIb) and an additional small peak (VIIc). Three of the 4 detected peaks could be related to compounds previously reported. The most abundant peak (VIIa) can be associated with 17α-methyl-17β-hydroxyandrost-1,4,6-trien-3-one (6-enemethandienone) (22, 24, 34). Two other peaks could be assigned to metabolite VII itself (17) and the 17-epimer of VII (VIIc) (27), by comparison to authentic reference standards. No metabolites have been found in the literature corresponding to peak (VIIb), and this peak was therefore considered to indicate a new unknown isomer of VII. Additional experiments will be necessary to elucidate the structure of this metabolite.

In the chimeric mouse urine only the epimer of VII (VIIc) could not be detected under the current instrumental conditions, but it should be noted that in humans VIIc is only a minor metabolite.

In the nonchimeric mouse only peak (VIIa), 6-enemethandienone, could be detected (Fig. 3). As already stated, the results for nonchimeric mouse samples correlate more with in vitro cultures because the detection of VIIa was previously reported in a study with cultured bovine hepatocytes (34). These results indicate that the use of the chimeric mouse model for validation already has contributed to knowledge of the metabolism of a previously intensively studied steroid, because 1 additional unreported isomer of VII was detected in the chimeric mouse model and was confirmed in humans.

In conclusion, we evaluated a uPA+/+ -SCID mouse model harboring human hepatocytes as an in vivo alternative for the investigation of steroid metabolism. Methandienone was administered to the mice as a steroid model compound. A validated LC-MS/MS method in mouse and human urine was developed. Application of the method to postadministration urines of mice and humans was shown for the quantification of 7 compounds, i.e., the parent methandienone and 6 metabolites. Comparison between the results obtained for humans and chimeric and nonchimeric mice confirmed that the chimeric mouse can be a useful model for the in vivo study of metabolic pathways of 17-alkylated steroids.

In the chimeric mouse 6 of the 7 human methandienone metabolites could be detected (6 of 7). The quantification also showed the differences between chimeric and nonchimeric control mice. In control mice only methandienone (I) and 6β-hydroxymethandienone (II) were detected (2 of 7), which correlates with the in vitro cultures in which 6β-hydroxylation was described as the major metabolic pathway.

Additional isomers of methandienone metabolites were also identified in both human and chimeric mouse urine. Three of the 4 isomers of metabolites VI and VII have previously been described or were confirmed by an available reference standard, but 1 detected compound has never been reported before.

The analysis of the postadministration urine samples resulted in the qualitative detection of 11 compounds in the human excretion urines and 9 compounds in the chimeric mouse urine (epimethiodiol (V) and
the epimer of VII, present in human urines, were not found in the chimeric mouse], whereas in the nonchimeric mouse urine only 3 compounds were found [the parent drug methandienone (I), 6β-hydroxymethandienone (II), and 1 additional iso-
mer of VII (VIIa)].

The chimeric uPA+/+–SCID mouse shows similar metabolic pathways to those in humans and can therefore be useful for in vivo confirmation of newly discovered steroid metabolites. The use of chimeric mice seemed to be a better and more complete approach than the traditional in vitro cultures.

The chimeric mouse model has some limitations, however, such as the low amount of urine excreted, the low absolute doses that can be administered, and the cost and complexity involved in the development of such a model with a high level of hepatocyte repopulation. Nevertheless, the chimeric mouse has demonstrated the ability to generate steroid metabolites in vivo that closely resemble those in the human profile. Further research is necessary with a larger mouse population and the use of more compounds to confirm the presence of all steroid metabolic pathways in the chimeric mouse model. In the future this promising model could potentially be used to identify metabolites of designer steroids. For this purpose sensitive open screening methods to detect unknown compounds are needed (35). The mouse model could also be applied for studies on drug metabolism and pharmacokinetics of new therapeutic agents or other pharmaceutical products.

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