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A Reproducible Method for the Identification of Methandrostenolone Metabolites Using a Humanized Liver Rodent Model

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ABSTRACT

Anabolic Androgenic Steroids (AAS), despite them being prohibited, are the most prevalent sport performance enhancing substances used by competing athletes. Identification of stable metabolites of AAS, such as methandrostenolone, in humans is required as reliable markers of their use. However, ethical issues make such studies difficult in human subjects. Therefore, the current study tested the hypothesis that a humanized urokinase Plasminogen Activator-Severe combined Immunodeficient murine model (uPA+/+-SCID mice) administered methandrostenolone at pharmacological concentrations, following both single and multiple dosing, is able to generate all its previously reported human metabolites. The hepatic mRNA expression of human Phase 1 enzymes was significantly altered in the drug treated chimeric mice, with an increase in aldehyde dehydrogenase activity being apparent in the serum. Following UHPLC/HRMS and GC/MSMS analysis of the urine, data showed the presence of all expected metabolites, including the long-term stable species, in the drug-treated, but not vehicle-treated, cohort. None of these changes were seen in the non-chimeric mice. Thus, this report provides further support for the reliability and sensitivity of uPA+/+-SCID mouse model for the identification of transient and stable metabolites of orally administered AAS in humans, especially following multiple dosing. The application of non-targeted metabolomics to samples derived from this model is likely to lead to the identification of novel metabolites of emerging, orally ingested anabolic agents.

Introduction

Anabolic Androgenic Steroids (AAS) are synthetic testosterone derivatives that increase and reduce muscle and fat mass respectively, making them the most prevalent sport performance enhancing substances used by both elite and recreational athletes [1]. Despite being prescription-only drugs, with even their recreational use being illegal in many countries, AAS derivatives are freely available and may be purchased in their pure form or as additives to supplements through numerous online outlets. Their effects on sport performance, and even more worryingly the adverse consequences of their chronic inappropriate use to general health, is very poorly understood. Therefore, there is pressure on anti-doping laboratories world-wide to develop reliable, reproducible and sensitive tests for the detection of both the parent AAS, as well as its stable metabolites [2]. One way of producing such tests is to screen for the metabolites of these steroids and identify the ones for use as markers. However, as these drugs are banned substances, ethical issues often make studies in human subjects difficult. The challenge to identifying metabolites for the various AAS and other related sport performance enhancing substances is the availability of an appropriate experimental model.

Several methods to produce these metabolites, both *in vitro* and *in vivo*, have been described [3]. While *in vitro* methods using human hepatocytes have been partially successful, they lack the whole-body environment to generate a full complement of metabolites. Reports on humanized-liver chimeras, where human hepatocytes are transplanted into the spleen of various mice, such as the uPA$^{+/+}$-SCID, Alb-TRECK/SCID and Por-deficinet PIRK mice models, are available [4-7]. These mice have been used as *in vivo* models to predict drug metabolism, pharmacokinetics and efficacy in humans. uPA$^{+/+}$-SCID chimeric mouse with highly humanized liver used in this study has been widely tested and validated in various applications more than the other aforementioned models [7]. Methandrostenolone, (metandienone/methandienone/17α-methyl-δ^{1}-testosterone/17α-methylandrost-1,4-dien-17ß-ol-3-one), a 17α-methylated derivative testosterone, whose metabolism has been most characterized, is an orally active, synthetic AAS. It is also known by its Ciba trade name, Dianabol. This is a popular AAS used for 'bulking up', by producing a rapid increase in muscle mass. Daily doses have been reported as being between 20-25 mg, taken for 5 days per week, over a 5-week cycle. It has a half-life of 3.5 hours, with systemic peak levels being reached between 1.5 to 3 hours after ingestion [8]. The primary urinary metabolite of methandrostenolone was reported to be detectable for up to 3 days, with the hydroxymethyl and sulphate metabolites for up to 19-26 days following a single oral (5mg or 25mg respectively) dose [9,10]. Many of these metabolites are unique to methandrostenolone. The current study aimed to confirm previous findings of urinary metabolites of orally administered methandrostenolone generated by the uPA$^{+/+}$-SCID mice transplanted with human hepatocytes, using pharmacological concentrations of the drug, both as single and multiple doses [5]. Changes in the hepatic mRNA expression of drug-metabolizing enzymes, and the aldehyde dehydrogenase activity in the serum, prior to and after administration of the drug, were also determined.

Material and Methods

Animal Studies

All *in vivo* experiments and procedures were carried out at KMT Hepatech lnc. (Edmonton, Canada) and approved by the Laboratory Animal Ethics Committee. uPA+/+-SCID chimeric mice with highly humanized liver were generated [11] where primary human hepatocytes were transplanted into the uPA$^{+/+}$-SCID mice and human albumin concentration was determined prior to drug administration to confirm the engraftment of human hepatocytes. uPA$^{+/+}$-SCID mice without transplanted human hepatocytes (non-chimeric mice) served as control animals. Chimeric mice were treated with a single or multiple dose of methandrostenolone (n=6) (Toronto Research Chemical, Canada) or 5% ethanol/PBS vehicle (n=6). Similarly, non-chimeric mice were also treated with methandrostenolone (n=4) or 5% ethanol/PBS vehicle (n=4). Methandrostenolone was administered by oral gavage (100µl of 400mg/L, 40µg/gavage) on day 0 (single dose: Figure 1a), and on days 0, 1 and 2 of the experiment (multiple doses: Figure1b). Metabolic cages were used to collect urine samples noninvasively from all animals prior to and on Days 1 and 4 after drug administration. Samples were stored at -20°C. All animals were sacrificed by Schedule 1 protocol at the end of the experiment. Blood was obtained by cardiac puncture without anticoagulant. Livers were harvested and flash frozen. Samples (urine, serum and livers) were collected.

RNA Extraction and CDNA Synthesis

Frozen liver tissue was ground to a fine powder using a pre-chilled mortar and pestle, with occasional addition of liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen, USA), precipitated with isopropanol (Sigma Aldrich, Germany) and washed with 70% ethanol (Sigma Aldrich, Germany). RNA quality was determined by 260:280nm ratio (NanoDrop, Thermo Scientific) and gel electrophoresis (Bioanalyzer, Agilent). RNA Integrity Number (RIN) of 5.5 was considered acceptable. cDNA was synthesized using first strand cDNA synthesis kit (Qiagen, UK).

Microarray Analysis

Pathway specific mRNA PCR arrays (RT2 profiler array Human/Mouse Drug Metabolism: Phase I Enzymes PCR Array, Qiagen) were

used to assess the expression of 84 key Phase 1 drug metabolism genes by real time PCR. Endogenous reference RNA controls were used to normalize the amount of target gene for relative quantification. Human and mouse arrays were used according to manufacturer's recommendations.

RT-PCR

Transcripts encoding genes identified in the arrays were validated by real-time PCR using ABI SYBR Green master mix (ViiA7 Applied Biosystems, USA). Specific primers were obtained from Qiagen (Qiagen, UK). The level of β-actin in each sample was used to normalize for the variability in RNA quantity. Data was presented as fold change (2-ΔΔCt) after normalization.

Aldehyde Dehydrogenase Activity in Serum

Serum was used to assess NAD-dependent Aldehyde Dehydrogenase (ALDH) activity. ALDH superfamily (19 genes) are key phase 1 cellular detoxification enzymes that oxidize various aldehydes and generate the corresponding carboxylic acid. It comprises 3 major classes: Class 1 and Class 3 are cytosolic and include both constitutive and induced forms; Class 2 is a constitutive, mitochondrial form. The ALDH activity was assayed using a fluorometric method (BioVision's PicoProbe, USA). ALDH is oxidized by acetaldehyde forming NADH that incorporates to the PicoProbe generating fluorescence (Ex/Em = 535/587) with sensitive detection of < 0.05 mU ALDH activity.

Urine Metabolite Analysis

All urine samples collected from the $uPA^{+/+}$ -SCID mice were analyzed by UHPLC/HRMS and GC/MSMS for the detection of the parent drug and its major metabolites. Additionally, a blank human urine sample spiked with reference standards of the above metabolites was analyzed to confirm the presence of the detected metabolites.

Sample Preparation

To 0.5 mL of urine, 1 mL of phosphate buffer, pH7, 50 µL of E-Coli β-glucuronidase (Sigma Aldrich) and 50 µL of a methanolic solution of internal standards mixture were added and the mixture was hydrolyzed at 50°C for 1.5 hours. The pH was then adjusted to 9 to 10 with sodium hydrogen carbonate and potassium carbonate (2:1) (w/w followed by liquid–liquid extraction against diethyl ether. The organic layer was separated from the aqueous phase after centrifugation by freezing and evaporation under nitrogen at 50°C and residue was reconstituted in solvent and injected to the UHPLC/HRMS system. Prior to GC/MSMS analysis, a derivatization step was performed to the dry residue by adding MSTFA/Ammonium iodide/2-Propanthiol (500:2:4) and incubation at 100°C for1 hour.

UHPLC/HRMS Conditions

A Dionex UHLC system (Thermo Scientific) was used for the chromatographic separation and the separation was achieved at 40°C using a Zorbax Eclipse Plus C18 column (Agilent Technologies). A gradient elution program was employed at a constant flow rate of 0.2 mL min-1 with injection volume of 5µL. The high resolution mass spectrometry was performed using QExactive Orbitrap mass spectrometer (Thermo Scientific). An additional targeted MSMS acquisition (product ion scan mode) at 17,500 resolving power was applied for the identification of the parent compound of methandienone(17α-methylandrost-1,4-dien-17β-ol-3-one) (precursor ion m/z 301.22 at normalized collision energy (NCE) 25%) and its metabolites 17-Epimethandienone (precursor ion m/z 301.22 at NCE 25%) and 18-nor-17β-hydroxymethyl,17α-methylandrost-1,4,13-trien-3-one (precursor ion m/z 299.20 at NCE 25%).

GC/MSMS Conditions

The GC/MSMS analysis was performed using Agilent GC 7890 system coupled with Agilent 7000C triple quadrupole mass spectrometer, equipped with a 7693 autosampler, 10µL syringe and split/splitless injector. Chromatographic separation was achieved using a SGE BPX5 column. The oven temperature program was set as ; 160°C for 0 min, ramped at 10°C /min up to 200°C, then ramped at 2 °C /min to 220 °C, ramped at 6 °C /min to 292 °C, 50°C /min up to 310°C and held for 3min. Injection volume was 2 µL in a split ratio 1:10 and the flow of the helium as carrier gas was 1.1 ml/min. The MS system was a QQQ with Electron Ionization at 70eV and multiple reaction monitoring (MRM) acquisition for the detection of Methandienone and its metabolites.

Statistical Analysis

Data were analyzed using Excel or by SA Biosciences in-house software. Significance was defined as $p \leq 0.05$.

Results

Microarray Analysis

In the animals that had been successfully transplanted with human hepatocytes (significantly elevated systemic human albumin) there was a global up-regulation of the Phase 1 metabolism enzymes in response to methandrostenolone. Sixty five percent of the genes in the Drug Metabolism: Phase I Enzymes array were induced after drug treatment as opposed to 52% following vehicle, this was particularly apparent when looking at genes that showed Ct between 25-30 (Figure 2a). The non-chimeric mice did not mount a xenobiotic response to the drug, probably because their residual liver function was compromised (Figure

2b). single and multiple dosing produced different patterns of gene expression (Figure 2c). More detailed analysis, using ß-actin, B2M and HPRT1 as the house-keeping genes for normalization and excluding samples that did not pass the QC checks, showed the mRNA expression of following genes to be significantly induced by the drug, compared to vehicle: Aldehyde dehydrogenase 7 family, member A1 (ALDH7A1) (1.33 fold difference, 95 % CI 1.27, 1.39: p=0.0003), Aldehyde dehydrogenase 9 family, member A1 (ALDH9A1) (2.26 fold difference, 95 % CI 0.79, 3.73: p=0.04) and Aldehyde dehydrogenase 6 family, member A1 (ALDH6A1) (1.88 fold difference, 95 % CI 0.70, 3.06: p=0.05).

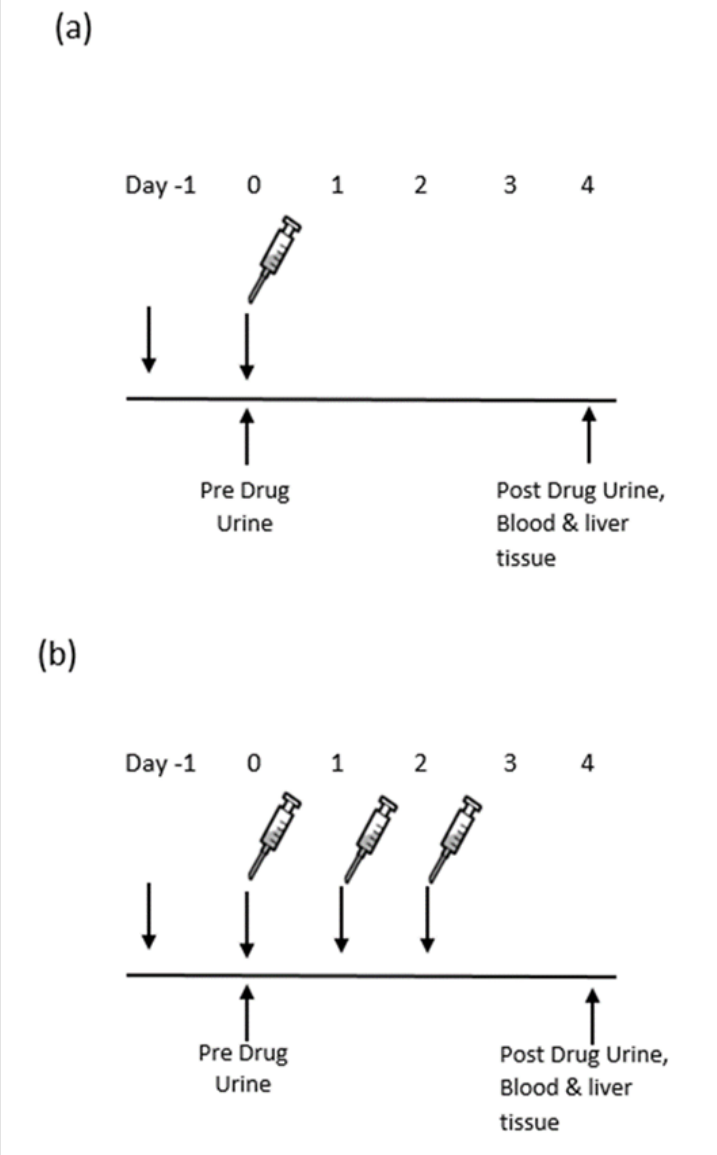

Figure 1: Experimental design and sample collection. Male uPA+/+-SCID chimeric (n=12) and non-chimeric (n=8) mice were administered with 100µl of 400mg/L (40µg/gavage) methandrostenolone by oral gavage, either as a single dose (a) or as multiple dose (b). 24h urine collections were made prior to and after drug administration on Days 0, 1& 4 of the experiment.

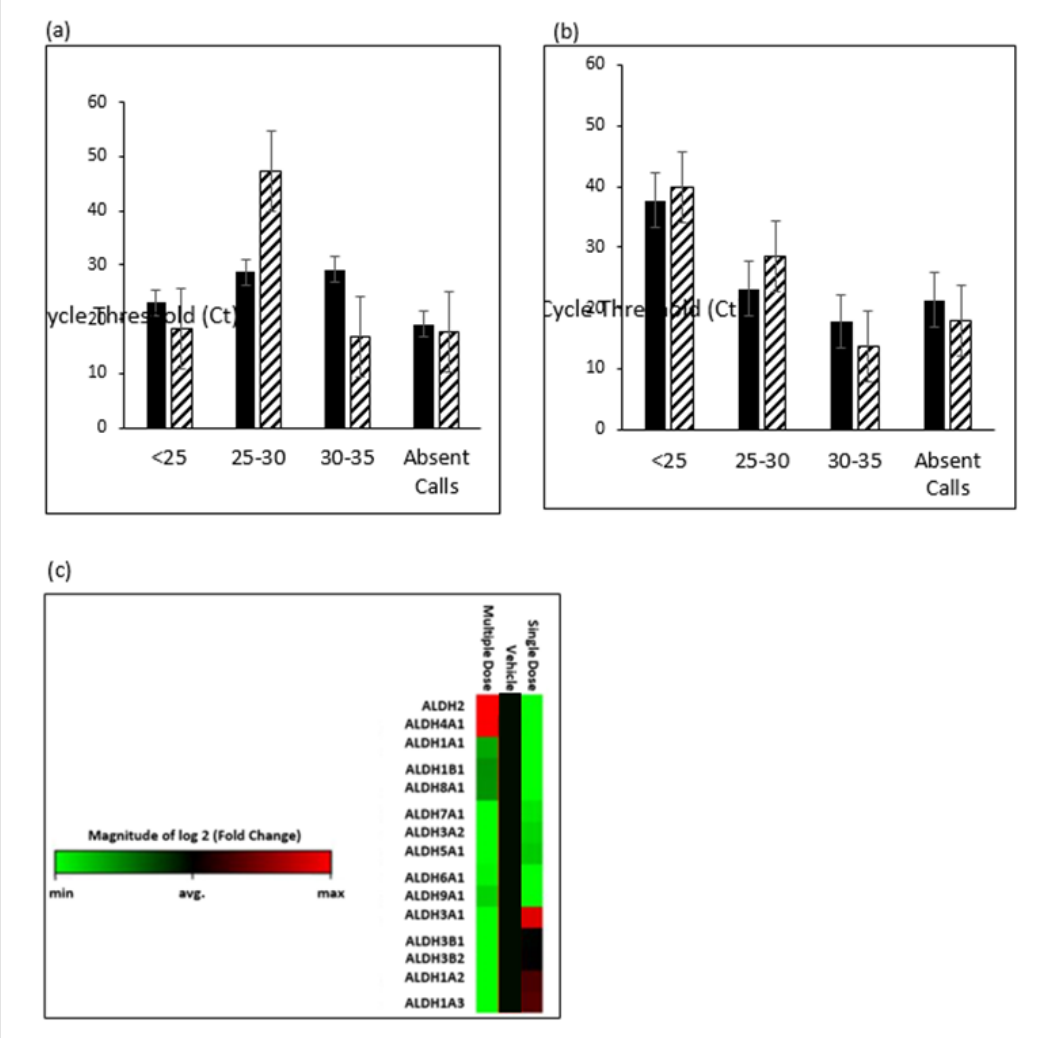

Figure 2: Up-regulation of hepatic mRNA expression of Phase 1 enzymes.

a) The mRNA expression of a significant number of the phase I enzymes were up regulated following injection with the drug, compared to vehicle, in the liver of chimeric mice using a human array (Solid bars – Vehicle, Hatched bars - Drug).

b) No such drug effect was seen in the non-chimeric mouse liver using a mouse array (Solid bars – Vehicle, Hatched bars - Drug), suggesting the lack of functionality of the organ in the host.

c) Heat map depicting magnitude of change in mRNA expression. Red=up-regulation; Green=down-regulation. Min=-2; Max=+2. n=6.

Aldehyde Dehydrogenase Activity in Blood

ALDH activity in serum was investigated in response to methandrostenolone using a high sensitivity fluorescent kinetic assay (NADH range 1-5 pmol/µl). Levels of ALDH activity in the samples was extremely low, but detectable. While there was an increase in the serum ALDH activity after the multiple, compared with the single, dose, this did not reach significance {Single 1.32 (0.6) versus multiple 1.56 (0.5) pmol/µl; p=0.45}.

Urine Metabolite Analysis

GC/MSMS detected17α-hydroxy-17ß-methylandrosta-1,4-dien-3-one , 17ß-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one and 17ß-hydroxy-17α-methylandrosta-1,4-dien-3-one with the rest by UHPLC/HRMS . The metabolites in the urine samples of the drug-treated animals are summarized in (Table 1). A comparison between single and multiple dosing showed that the latter mainly increased the sensitivity of the detection of all the studied metabolites. None of the mentioned

metabolites was detected in the vehicle treated chimeric mice, after single or multiple doses. Following UHPLC/HRMS and GC/MSMS analysis of the urine, data showed the presence of all the previously reported metabolites [4,6], including the stable metabolite, in the drug-treated cohort but not in those treated with vehicle (Table 2).

Table 1: Detected metabolites using GC/MSMS and UHPLC/HRMS. As the analysis was qualitative, data are shown as (-) or (+), with (-) as not detected and variable detection accuracy of 33.3% (+), 66.6% (++) and 99.9% (+++).

Analytes	Single Dose			Multiple doses		
	Predrug	24hrs	96hrs	Predrug	24hrs	48hrs
17α-methyl-5β-androstane-3α,17β-diol	-	-	+	-	+	+++
17α-hydroxy-17ß-methylandrosta-1,4-dien-3-one,	-	++	+	-	-	+++
17ß-hydroxy-17α-methylandrosta-1,4-dien-3-one	-	++	+	-	-	+++
6ß,17ß-dihydroxy-17α-methylandrosta-1,4-dien-3-one	-	++	++	-	++	+++
17ß-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one	-	+	-	-	-	+
16α,17ß-dihydroxy-17α-methylandrosta-1,4-dien-3-one	-	+	-	-	++	+++
18,17ß-dihydroxy-17α-methylandrosta-1,4-dien-3-one	-	+	-	-	-	+++

Table 2: Comparison of metabolites detected in the current versus previous study [5].

Metabolites	Lootens, et. al [5]	Current study
17,17-dimethyl-18-nor-5ß-androsta-1,13-dien-3α-ol	ND	ND
17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one	D	ND
17ß-methyl-5ß-androst-1-en-3-3α,17α-diol	ND	ND
17α-methyl-5β-androstane-3α,17β-diol	ND	D
17α-hydroxymethyl-17β-methyl-18-norandrosta-1,4,13-trien-3-one	ND	ND
17α-hydroxy-17ß-methylandrosta-1,4-dien-3-one	D	D
17ß-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one	D	D
17ß-hydroxy-17α-methylandrosta-1,4-dien-3-one	D	D
6ß,17ß-dihydroxy-17α-methylandrosta-1,4-dien-3-one	D	D
18,17ß-dihydroxy-17α-methylandrosta-1,4-dien-3-one	Not assayed	D
16α,17ß-dihydroxy-17α-methylandrosta-1,4-dien-3-one,	Not assayed	D

Note: D: Detected; ND: Not detected.

Discussion

Determination of AAS use in the human sporting arena has been, and continues to be, a significant challenge to scientists. Using a well characterized AAS, methandrosternolone, this study confirmed that the uPA+/+-SCID model with the humanized liver is capable of generating all previously reported urinary metabolites. Furthermore, oral administration of the drug over 3 days (Figure 1) provides a valid experimental set up. Also, both multiple doses, as well as a single dose, leads to detectability of the reported urinary metabolites, as well as elevated mRNA expression of hepatic phase I enzymes. The multiple doses, however, increased the sensitivity of the detection, especially of specific aldehyde dehydrogenases and cytochrome P450. Whilst ALDH activity was detectable in the serum it did not reach significance in the presence of drug and perhaps best investigated in hepatic tissue. Previous studies have also used the same rodent model [5,12], however, with different derivatives of testosterone (4-androstene-3,17-dione and 17α-methyltestosterone respectively; (Figure 3), concentrations and the numbers of doses. The study by Pozo et al. used 17α-methyltestosterone following a single dose of the drug (40 µg per oral gavage) [12]. On the other hand, Lootens et al. used multiple, supratherapeutical doses (350µg per gavage) of 4-androstene-3,17-dione [5]. The current study used both single and multiple doses, at pharmacological concentrations (40 µg) of methandrosternolone. The multiple doses increased the sensitivity of detection, despite all the reported metabolites being detectable upto 96h after a single dose.

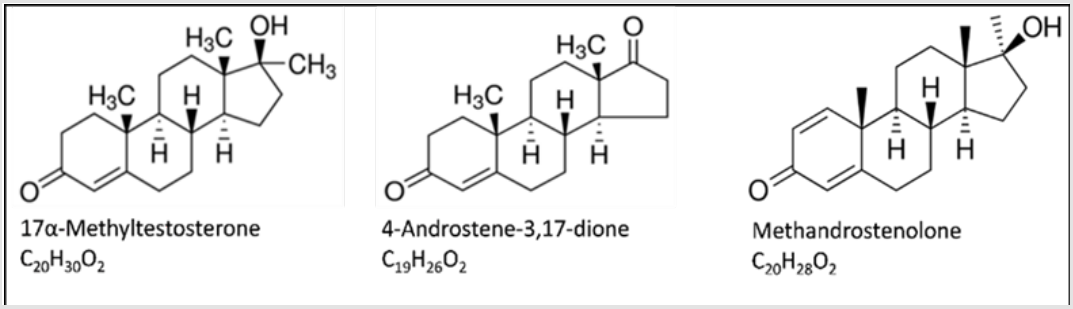

Figure 3: Testosterone derivatives used to investigate human drug metabolism in uPA+/+-SCID mice. Left panel: Derivative used by Pozo, et. al [12]; Middle panel derivative used by Lootens, et. al [5]; Right panel derivative used in current study.

This report clearly provides further support for a reliable *in vivo* murine model for the identification of transient and stable metabolites of orally administered AAS in humans, especially following multiple dosing. Humanized liver chimeric mice were validated against an array of xenobiotics and were able to be metabolically distinct from recipient mice, while showing metabolic pathways analogous to those expected from human liver. Recent metabolic studies in the chimeric uPA+/+-SCID mouse model, including steroid metabolism, were reviewed in Foster et al [7]. The model closely reflects the human metabolism of some drugs, as well as predict human metabolites, including those generated through more than one metabolism reaction [13]. Using both UHPLC/HRMS and GC/MSMS the current study confirmed detection of previously reported metabolites of related testosterone derivatives. While this study used a targeted approach, it may prove very interesting when combined with non-targeted metabolomics for the identification of novel metabolites, especially for emerging anabolic agents.

Data Availability

Any data presented here will be available by email through the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

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