Dimethandrolone (DMA), a Potential Male Contraceptive Pill, is Primarily Metabolized by the Highly Polymorphic UGT2B17 Enzyme in Human Intestine and Liver

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Abbreviations: DMAU, dimethandrolone undecanoate; DMA, dimethandrolone; TG, testosterone glucuronide; 17βHSD2, hydroxysteroid 17-β dehydrogenase 2; UGT2B17, UDP-glucuronosyltransferase 2B17; CYP3A4, cytochrome P450 3A4; DMA-G, DMA glucuronide; PK, pharmacokinetics; LC-HRMS, liquid chromatography-high resolution mass spectrometry; HIM, human intestinal microsomes; HLM, human liver microsomes; rhUGT, recombinant human UGT; \(f_m,UGT2B17\), drug fraction glucuronidated by UGT2B17; CNV, copy number variations.

Keywords: UGT2B17, Dimethandrolone, Inter-individual Variability, Male Contraceptive
ABSTRACT

Dimethandrolone undecanoate (DMAU), an oral investigational male hormonal contraceptive is a prodrug, which is rapidly converted to its active metabolite, dimethandrolone (DMA). Poor and variable oral bioavailability of DMA after DMAU dosing is a critical challenge to develop it as an oral drug. The objective of our study was to elucidate the mechanisms of variable pharmacokinetics (PK) of DMA. We first identified DMA metabolites formed \textit{in vitro} and \textit{in vivo} in human hepatocyte incubation and serum samples following oral DMAU administration in men, respectively. The metabolite identification study revealed two metabolites, DMA-glucuronide (DMA-G; major) and the androstenedione analogue of DMA (minor) in the hepatocyte incubations. After oral DMAU administration, only DMA-G was detected in serum, which was >100-fold compared to DMA levels, supporting glucuronidation as the major elimination mechanism for DMA. Next, thirteen clinically-relevant UDP-glucuronosyltransferase (UGT) enzymes were tested for their involvement in DMA-G formation, which revealed a major role of UGT2B17 isoform with a smaller contribution of UGT1A9 in DMA-G formation. These data were confirmed by dramatically higher DMA glucuronidation rates (>200- and >7-fold) in the high versus the null UGT2B17-expressing human intestinal and liver microsomes, respectively. Since human UGT2B17 is a highly variable enzyme with a 20-80% gene deletion frequency, the \textit{in vitro} data suggest a major role of UGT2B17 polymorphism on the first-pass metabolism of DMA. Further, considering DMA is a selective and sensitive UGT2B17 substrate, it could be used as a clinical probe of UGT2B17 activity.
SIGNIFICANCE STATEMENT

Dimethandroline (DMA) is an active metabolite of dimethandroline undecanoate (DMAU), an investigational male hormonal contraceptive. Previous studies have indicated poor and inconsistent bioavailability of DMAU following oral administration. Our study found that UGT2B17-mediated high intestinal first-pass metabolism is the key mechanism of variable DMA bioavailability.
INTRODUCTION

In the United States, only 30% of couples use a male method of contraception, with 20% using condoms for contraception and 10% relying on vasectomy (Daniels et al., 2015). This necessitates the need for developing a safe and reversible male contraceptive to reduce the disparity in contraception use and to encourage equal participation of both the sexes in family planning (Amory, 2020). Ideally, an oral male pill could offer the most convenient and accessible contraceptive option to men (Weston, et al., 2002). Unfortunately, attempts to use oral testosterone as contraceptive failed because it is cleared too rapidly as a single daily dose regimen even in combination with progestin analogues, whereas multiple doses of oral testosterone per day would be impractical for contraception. Although 17-methyltestosterone has good oral bioavailability, its long-term use has been associated with hepatotoxicity (Westaby et al., 1977). Thus, an oral male contraceptive with a longer duration of action is an unmet need.

Dimethandroline undecanoate (DMAU; 7α,11β-Dimethyl-19-nortestosterone 17β-undecanoate) is a promising experimental male hormonal contraceptive. DMAU is a prodrug that is metabolized to its active metabolite, dimethandroline (DMA) (Surampudi et al., 2014; Ayoub et al., 2017; Thirumalai et al., 2019) (Figure 1A). DMA binds to both the androgen and the progesterone receptors, and hence can be potentially used as a single-agent male contraceptive (Attardi et al., 2011) (Figure 1B). However, poor and variable oral bioavailability of DMAU (and DMA) including the first-pass metabolism and positive food-effect (13- and 6-fold AUC of DMAU and DMA in fed compared to fasting states) (Surampudi et al., 2014; Ayoub et al., 2017) is a critical challenge affecting its potential success as an oral drug. Moreover, DMAU and DMA are highly lipophilic (logP 6.5 and 3.9, respectively) and poorly water soluble (4.4 × 10^{-5} and 0.0293 mg/ml, respectively) (Attardi et al., 2011; Ayoub et al., 2017). Poor solubility could be a possible reason for the poor DMA bioavailability, however, the role of first-pass metabolism...
of DMA has not been ruled out. In particular, DMA and testosterone are structurally similar (Figure 1C), and importantly, DMA retains the 17β-hydroxy group, and therefore it is highly susceptible to glucuronidation in the intestine and liver. Testosterone is extensively metabolized in human enterocytes and hepatocytes to multiple primary metabolites including androstenedione, testosterone glucuronide (TG), and 6-hydroxy testosterone, by hydroxysteroid 17-β dehydrogenase 2 (17βHSD2), UDP-glucuronosyltransferase 2B17 (UGT2B17), and cytochrome P450 3A4 (CYP3A4), respectively (Zhang et al., 2018). Among these metabolites, TG is one of the predominant metabolites after oral administration with >80-fold exposure in men compared to testosterone levels and UGT2B17 is the main enzyme involved in testosterone to TG conversion (Basit et al., 2018). UGT2B17 is predominantly expressed in human intestine than in the liver, and copy number variation (CNV) in its gene has been shown to be associated with inter-individual variability in urinary excretion of TG (Jakobsson et al., 2006; Schulze et al., 2008) and drug metabolism including first-pass metabolism (Wang et al., 2012; Basit et al., 2018). Therefore, we hypothesized that extensive first-pass metabolism of DMA is the primary mechanism of its poor and variable bioavailability, and UGT2B17 is the major enzyme that converts DMA to DMA glucuronide (DMA-G). We investigated the in vitro and in vivo metabolic profiling of DMA to elucidate the mechanisms leading to its variable pharmacokinetics (PK). First, we identified DMA metabolites formed in human hepatocyte incubation and in serum samples after oral DMAU dosing by liquid chromatography-high resolution mass spectrometry (LC-HRMS). Second, we elucidated UGT isoforms involved in DMA glucuronidation. Finally, we determined the effect of UGT2B17 gene deletion on DMA metabolism in human intestinal and liver microsomes (HIM and HLM, respectively) and quantified the fraction of DMA glucuronidation by specific UGTs (f_m) in the intestine and liver. The in vitro findings were confirmed through in vivo PK analysis of human serum samples from an oral PK study of DMAU. Overall, this mechanistic study explains challenges with oral
bioavailability of DMA and provides insights into developing better approaches for DMAU oral delivery.

MATERIALS AND METHODS

Chemicals, reagents, and software

DMAU, DMA and their respective stable-labeled (d5-deuterated) standards were obtained through Dr. Min Lee (Eunice Kennedy Shriver National Institute of Child Health and Human Development, NICHD) from Ash Stevens (Riverview, MI). LC-MS grade acetonitrile (MeCN), methanol, chloroform, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Alamethicin, UDP-glucuronic acid (UDPGA), magnesium chloride, dibasic and monobasic potassium phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium bicarbonate (ABC) (98% pure), bovine serum albumin (BSA), dithiothreitol (DTT), iodoacetamide (IAA), and trypsin were procured from Thermo Fisher Scientific (Rockford, IL). Oasis HLB cartridges were purchased from Waters (Milford, MA). Testosterone, TG and their respective stable-labeled (d3-deuterated) standards were purchased from Cerilliant Corporation (Round Rock, TX). Male human plateable cryopreserved hepatocytes (Product # M00995; n=3) were obtained from BiolVT (Westbury, NY), and hepatocyte thawing (HT) and hepatocyte incubation (HI) media were procured from Invitrogen (Waltham, MA). Recombinant human UGT (rhUGT) enzymes were procured from Corning life sciences (Corning, NY). Pooled HIM and HLM (mix of male and female subjects) were obtained from Xenotech (Kansas City, KS). Individual genotyped and de-identified (i.e., identifiable donor information such as donor name was unknown to us). HLM samples were from our previous study (Zhang et al., 2018) that were procured from the University of Washington School of Pharmacy Liver Bank (Seattle, WA). Synthetic light (with amino acid analysis) and stable isotope-labeled peptides were purchased from New England Peptides (Boston, MA) and Thermo Fisher Scientific (Rockford, IL), respectively. MATLAB (Natick, MA), BioRender (Toronto, Ontario), GraphPad (San Diego, CA),
DMA metabolism in human hepatocytes

Cryopreserved human hepatocytes were thawed and plated according to a previously defined protocol (Basit et al., 2018). Briefly, the HT and HI media were warmed at 37 °C for 30 min. The hepatocyte vial was completely thawed by immersing into a 37 °C water-bath. Thawed hepatocytes were poured gently into the HT medium in a 50 ml conical tube, mixed by gently inverting few times, and centrifuged at 100 x g for 10 min at room temperature. The cell pellet was gently resuspended in 3 ml of HT medium, and hepatocytes were counted using the Trypan Blue exclusion method using Auto T4 cellometer (Nexcelom Bioscience, Lawrence, MA). HI medium was added to the cell suspension to make 1 x 10^6 cells per ml concentration. The reactions were initiated by adding 500 µl of hepatocyte suspension to 500 µl pre-warmed HI medium containing 5 µl of 15, 2 and 0.2 µM DMA and DMAU separately, in 6-well plates (n=3 individual hepatocytes) (Table S1), i.e., final cell count of 0.5 x 10^6 cells per well and a final concentration of 0.1, 10 and 75 µM DMA. The 6-well plates were kept into a 37 °C incubator maintained at 5% CO₂ immediately after seeding. The reaction was quenched with 5-fold higher volume of ice-cold MeCN containing internal standard (heavy isotope labelled DMA-d5) compared to the total sample volume at 60 and 120 min. The sample was vortexed for 1 min to extract all the metabolites followed by centrifugation at 3000 x g for 10 min (4°C). The supernatant was subjected to freezing (-20 °C) till both MeCN, and aqueous layers became immiscible, and aqueous layer was frozen. The upper MeCN layer (drug and non-polar metabolites) were collected in another tube. The lower aqueous layer (polar metabolites) was subjected to solid phase extraction (SPE) using HLB cartridge. The procedure involved cartridge conditioning with 2 ml methanol followed by 2 ml water containing 0.2% formic acid, loading of samples (0.5 ml), washing with 1 ml water containing 2% methanol and 0.2% formic acid, and
elution of the analytes with 0.5 ml MeCN. Finally, aliquot from SPE was pooled with the previously collected MeCN layer. The mixtures were dried at 40 °C using a nitrogen evaporator (Techne, Minneapolis, MN), and the dried samples was reconstituted in 100 µl of 70:30 MeCN:water (0.1% formic acid) and centrifuged at 10,000 x g for 10 min. The supernatant was stored at -80 °C prior to LC-MS/MS analysis.

**DMA metabolism in human serum**

The representative serum samples (n=3 males) (Table S2) were available to us from a previous clinical study (Ayoub et al., 2017) conducted at the Lundquist Institute at Harbor-UCLA Medical Center. The clinical study was approved by the institutional review board and the study details are reported previously (Surampudi et al., 2014; Ayoub et al., 2017). The subjects were given a 100 mg dose of oral DMAU in castor oil with a high fat meal (>50% of fat, 800-900 calories, with approximately 150, 250 and 500-600 calories of protein, carbohydrates, and fat, respectively). For the metabolite characterization, a pooled sample (20 µl) at each time point, specifically 0.5, 1, 2, 4, 6, 8, 12, and 18-hour after oral administration was mixed with 90 µl of ice-cold MeCN containing 0.1% formic acid and 25 ng/ml of internal standard (DMA-d5). The sample was vortex mixed and centrifuged at 10,000 x g for 10 min (4 °C). Ten µl of the supernatant was collected and added with 50 µl of water containing 0.1% formic acid and transferred to an LC vial for LC-HRMS analysis.

**DMA metabolite identification in human hepatocyte and serum samples**

Prior to the analysis of DMA metabolites, a list of structurally feasible metabolites was generated using testosterone metabolic profile as described previously (Basit et al., 2018; Zhang et al., 2018) (Table 1). A targeted data dependent metabolomics approach was used to screen the predicted metabolites of DMA. Other than predicted metabolite structures of DMA, untargeted metabolomics was performed by comparing high resolution MS data of pre- and post-dose samples, using XCMS Online. The following screening criteria were employed to filter
potential metabolites: i) post-dose sample with >10-fold response compared to the pre-dose sample, ii) mass defect filter (mDA) range from -50 to 50, iii) retention time window of 20 to 46 min (considering highly polar DMA-G was observed at ~27 min), and iv) MS intensity of >10^6 in post-dose sample, and MS intensity of <10^6 in pre-dose sample.

Both in vitro (hepatocyte incubation) and in vivo (serum) samples were analyzed using Thermo Scientific™ Q Exactive™ HF with nano-flow LC in data dependent acquisition (DDA) mode. Chromatographic separation of metabolites was achieved using a Thermo reversed phase nano C18 column (75 × 250 mm, 2 μm particle size). LC conditions were set at 300 nl/min flow rate and 1 μl injection volume with mobile phase A: water with 0.1% formic acid, and B: MeCN with 0.1% formic acid. The gradient was as follows: 0.0-5.0 min (5% B), 5.0-20.0 min (5-50% B), 20.0-35.0 min (50-80% B), 35.0-45.0 min (80-80% B), 45.0-46.0 min (80-90% B), 46.0-56.0 min (90-90% B) and 56.0-57.0 min (90-5% B), followed by an 8 min gradient of 5% B. The mass spectrometer was operated in full MS and data dependent MS^2 in the positive polarity mode with a scan range of 250 to 1000 m/z and 200 to 2000 m/z for full MS and MS^2, respectively (Table S3).

**In vitro characterization of DMA glucuronidation enzyme**

**UGT screening assay of DMA**: Thirteen UGT enzymes were tested for their potential to form DMA-G. These enzymes include UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15 and 2B17. Briefly, 80 μl mixture of alamethicin (2 mg/ml), BSA (0.2%, 2 mg/ml), and 5 mM MgCl_2/100 mM potassium phosphate buffer (pH 7.4) (1:1:14, respectively) was added, followed by addition of 5 μl of 0.2 mg/ml rhUGT enzymes to 96 well plate. Next, 10 μl of DMA (100 μM) was added, and kept on ice for 15 min, followed by initialization of the reaction by adding 5 μl of 50 mM UDPGA, and incubation for 45 min (37 °C) at 300 rpm. The reaction was quenched using 200 μl ice-cold MeCN containing TG-d3 as internal standard. The samples
were centrifuged at 2,000 g for 5 min (4°C) and the supernatant was transferred to LC vial. The reaction was performed in triplicates.

**DMA-G assay in rhUGT2B17, HIM, and HLM:** DMA-G assay was performed in rhUGT2B17, HIM, and HLM. For DMA-G kinetic assays, similar experimental procedure was followed as discussed above with DMA concentration ranging from 0.2-100 μM. After 15 min of pretreatment on ice, reactions were initiated by addition of 5 μl of 50 mM UDPGA, incubated for 30 min (37°C) at 300 rpm, and reaction was terminated using 200 μl of ice-cold MeCN containing TG-d3. The samples were then centrifuged at 2,000 g for 5 min (4°C) and the supernatant was collected in LC vial for the analysis. The reaction was performed in triplicates.

**Preparation of stock solutions, calibration standards, and quality controls for analysis:** A mix of stock solutions of DMA and DMAU (1:10) was serially diluted into thirteen working standards in methanol (0.06-250 ng/ml and 0.61-2500 ng/ml, respectively). Ten μl working standard solution was spiked into 40 μl of blank biological matrix (human plasma: water, 50:50 v/v), and protein was precipitated by adding 200 μl of the internal standard (25 ng/ml DMA-d5 in methanol). The sample was vortex mixed for 15 sec followed by centrifugation at 15,000 x g (4°C) and the supernatant was collected for LC-MS analysis. Similarly, quality control (QC) samples representing three concentrations were prepared (low: 0.12 and 0.01 pg/ml; medium: 7.8 and 0.78 pg/ml; high: 500 and 50 pg/ml, DMAU and DMA, respectively).

Because DMA-G standard was not available, we utilized a surrogate standard method to analyze DMA-G in *in vitro* hepatocyte incubations and serum samples. Here, testosterone and TG calibration standards (2, 10, 25, and 50 ng/ml) were prepared in methanol and the ratio of TG/testosterone response was used as a surrogate of DMA-G/DMA response using identical MS parameters.
**LC-MS/MS and data analysis of in vitro DMA kinetics samples**: Samples were centrifuged at 10,000 g for 5 min (4°C) and the supernatant (150 µl) was collected and transferred to an LC vial for analysis by LC-MS/MS (Waters Xevo-TQ-XS MS; Waters, Milford, MA) with standard ESI source, microflow LC and Acquity UPLC HSS T3 column (100Å, 1.8 µm, 1 mm x 100 mm). LC conditions were set at 40 µl/min flow rate and 1 µl injection volume using mobile phase A: water with 0.1% formic acid and B: MeCN with 0.1% formic acid, using the following gradient program: 0.0-0.5 min (30% B), 0.5-3.5 min (30-98% B), 3.5-7.0 min (98-98% B), and 7.0-7.1 min (98-30% B), followed by a 1.9-min gradient of 30% B. The mass spectrometer was operated in multiple reaction monitoring (MRM) and positive ion (ESI+) mode with cone voltage (CV) of 30 V. The MRM transitions were: DMA-G (m/z 479.3→ 285.4 and 303.2; collision energy (CE) 25 eV), TG-d3 (m/z 468.2→ 97.1 and 109.1; CE 40 eV), testosterone (m/z 289.2→ 97 and 109; CE 40 eV), and TG (m/z 465.3→ 271.2 and 289.2; CE 25 eV).

Enzyme kinetic parameters (Michaelis-Menten constant, $K_m$ and maximal rate of velocity, $V_{max}$) were obtained by fitting the Michaelis-Menten equation using GraphPad Prism v. 8.4.3 (Equation 1), where $Y$ is DMA-G formation rate, and $X$ is DMA concentration.

\[
Y = \frac{V_{max} \times X}{(K_m + X)} \quad \text{Eq. 1}
\]

**LC-MS/MS protein quantification of UGT1A9 and UGT2B17 proteins in HIM and HLM**: We applied an optimized targeted LC-MS/MS methodology to selectively quantify UGT1A9 and UGT2B17 enzymes (Ahire et al., 2021). Peptide selection for the proteins was performed using a previously discussed *in silico* approach (Prasad et al., 2019). Total protein concentration was quantified using a BCA assay kit (Pierce Biotechnology, Rockford, IL) following the vendor protocol and the proteins were digested as described previously (Ahire et al., 2021). Briefly, 80 µl of the tissue sample (1 mg/ml total protein) was mixed with 30 µl ammonium bicarbonate (100 mM) and 20 µl of BSA (0.02 mg/ml) in a 1.5 ml micro-centrifuge tube. Proteins were denatured
and reduced with 10 μl of 250 mM DTT at 95 °C for 10 min with gentle shaking at 300 rpm. Sample was cooled at room temperature for 10 min, and the denatured protein was alkylated with 10 μl of 100 mM IAA in the dark for 30 min. One ml of ice-cold acetone was added to each sample followed by vortex-mixing and incubation at -80 °C for 30 min. Next, the sample was centrifuged at 16,000 × g (4 °C) for 10 min, the supernatant was carefully removed using vacuum suction. The protein pellet was dried at room temperature for 30 min and washed with 500 μl ice-cold methanol followed by centrifugation at 16,000 × g (4 °C) for 10 min. The supernatant was removed, the pellet was dried at room temperature for 30 min and re-suspended in 60 μl ammonium bicarbonate buffer (50 mM, pH 7.8). Finally, the reconstituted protein sample was digested by adding 20 μl of trypsin (protein: trypsin ratio, approximately 50:1) and incubated for 16 hours at 37 °C. The sample was centrifuged at 1000 × g (4°C) for 1 min and kept in -20 °C for 5 min. Then, the reaction was quenched by the addition of 10 μl of peptide internal standard cocktail (prepared in 80% MeCN in water containing 0.5% formic acid) and 5 μl of 5% formic acid in water. The sample was vortex mixed, and centrifuged at 4000 × g for 5 min. The supernatant was collected in a LC-MS vial for analysis. LC-MS/MS data acquisition was performed on an M-class Waters UPLC system coupled with Waters Xevo® TQ-XS microflow LC-MS/MS instrument connected to standard ESI source using optimized parameters outlined in Tables S4 and S5. The peptides were separated on standard HSS T3 C18 column (1.8 μm, 1.0 x 100 mm). All samples were digested and analyzed in triplicate. The proteotypic peptides of UGT1A9 (AFAHAQWK) and UGT2B17 (FSVGYTVEK and SVINDPIYK) were quantified in the digested samples using a validated LC-MS/MS method (Ladumor et al., 2019; Ahire et al., 2021). Light peptides were used as calibrators and the corresponding heavy peptides containing terminal labeled $^{13}$C$_6$ $^{15}$N$_2$-lysine or $^{13}$C$_6$ $^{15}$N$_4$-arginine residues served as internal standards.
Calculation of the fractional metabolism of DMA by UGT enzymes ($f_m$): The $f_m$ values were calculated using the $V_{\text{max}}$ values from high- and null-expressing HIM and HLM experiments (Equations 2 and 3).

$$f_{m,\text{UGT2B17}} = \frac{V_{\text{max, high UGT2B17 expressing HIM}} - V_{\text{max, null UGT2B17 expressing HIM}}}{V_{\text{max, high UGT2B17 expressing HIM}} + V_{\text{max, null UGT2B17 expressing HIM}}} \quad \text{Eq. 2}$$

$$f_{m, \text{non-UGT2B17}} = \frac{V_{\text{max, null UGT2B17 expressing HIM}}}{V_{\text{max, high UGT2B17 expressing HIM}} + V_{\text{max, null UGT2B17 expressing HIM}}} \quad \text{Eq. 3}$$

Analysis of DMA in human serum samples and PK analysis

Sample preparation and LC-MS/MS Analysis: We adopted a previously reported method (Surampudi et al., 2014; Ayoub et al., 2017) for DMA quantification in human serum samples with a few modification. Briefly, for the analysis of PK samples from three individuals, 25 µl of serum sample was diluted 2-fold with water and the internal standard (200 µl of 25 ng/ml DMA-d5) was added. The sample was vortex mixed for 15 sec followed by centrifugation at 15,000 x g for 10 min (4°C). The supernatant (150 µl) was collected and transferred to LC vial for analysis by LC-MS/MS (M-class LC coupled to Waters Xevo-TQ-XS) with standard ESI source and microflow LC.

The LC-MS conditions used for analyzing human serum samples were similar to that shown for in vitro DMA kinetics sample analysis above, except that the injection volume was 2 µl. For additional analytes, the MRM transitions were: DMA (m/z 303.2→ 97 and 109; CE 40 eV) and DMA-d5 (m/z 308.3→ 102 and 114; CV 30; CE 40 eV).

The method for DMA quantification was validated for linearity, dynamic range accuracy, and analytical range. The method was linear ($r^2 = 0.9956$) with the accuracy of 97%, 112%, and 106% corresponding to DMA concentrations of 0.8 ng/ml (low concentration QC; LQC), 6.25 ng/ml (medium concentration QC; MQC) and 12.5 ng/ml (high concentration QC; HQC), respectively. The lower limit of quantification (LLOQ) was 0.4 ng/ml, and the upper limit of
quantification (ULOQ) was 50 ng/ml. The response of the stable isotope labeled internal standard (DMA-d5) was consistent across samples. Because the synthetic standard of DMA-G was not available, a surrogate standard approach was used for DMA-G quantification (Equation 4). The ratio of TG to testosterone was used for DMA-G quantification assuming that the glucuronidation results in similar changes in the mass ionization efficiencies of DMA and testosterone assuming that the critical LC-MS/MS parameters remains similar between the pairs (afore-mentioned).

\[ [\text{DMA} - G \text{ concentration}] = [\text{DMA concentration}] \times \frac{\text{TG MS response}}{\text{Testosterone MS response}} \]

**Eq. 4**

**Pharmacokinetic data analysis:** The calculation of PK parameters was performed via MATLAB R2021b software through noncompartmental PK analysis. All data were expressed as mean ± standard deviation (SD).

**RESULTS**

**Metabolite characterization of DMA in human hepatocyte and serum samples**

We first leveraged testosterone metabolic profile to predict the plausible structures of DMA metabolites. Out of 21 structurally feasible metabolites of DMA, only DMA-G (m/z 479.2639) and androstenedione analogue of DMA (m/z 301.2162) were formed in hepatocyte incubations, but only DMA-G was detected in human serum samples following an oral dose of 100 mg DMAU (Table 1 and Figure S1). In addition, differences in metabolomics profiles of pre- and post-dose serum samples were evaluated using XCMS software, which further confirmed DMA-G as the predominant metabolite. Although untargeted metabolomics data showed some additional species (Table S6), they were either found in both pre- and post-dose samples, small in abundance, or were potentially endogenous metabolites likely affected by the treatment. Interestingly, other predicted metabolites based on testosterone metabolite profile were not detected (or were below limit of quantification) following DMA or DMAU incubations. The LC-
HRMS analysis of human hepatocyte and serum samples confirmed that glucuronidation is the major biotransformation pathway involved in DMA metabolism (Figure S1). No DMA was detected at 1 µM DMA incubation, suggesting its rapid elimination to DMA-G. The ratio of DMA-G to DMA concentrations were ~ 2-fold at 10 µM hepatocyte incubations, confirming higher formation of the metabolite compared to the parent (Figure 2). In addition, both DMA-G and androstenedione analogues to parent ratios were comparable in both DMA and DMAU incubations, indicating that DMAU to DMA formation is not the rate-limiting step (Figure S2).

**UGT screening assay of DMA**

Out of the thirteen clinically relevant rhUGT enzymes (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, UGT2B15 and 2B17) that were tested for their capacity to metabolize DMA, only UGT2B17 and UGT1A9 were found to metabolize DMA to DMA-G. DMA-glucuronidation rate was 10-fold in rhUGT2B17 as compared to in rhUGT1A9 incubations (Figure 3A). The commercial rhUGT2B15 enzyme was not active against its probe substrates (oxazepam, data not shown), thus the role of UGT2B15 could not be measured using the recombinant system.

**Enzyme kinetics parameters for DMA in rhUGT2B17, HIM and HLM**

DMA-G formation kinetic analysis in rhUGT2B17 showed $V_{\text{max}}$ of 8 nmol/min/mg protein and the $K_m$ of 1.63 µM (Figure 3B). The kinetic parameters, i.e., $V_{\text{max}}$ and $K_m$ values for DMA-G formation in the high and null UGT2B17-expressing HIM were: 19.14 and 0.07 nmol/min/mg protein, and 3.4 and 12.7 µM, respectively (Figure 3C). Similarly, the $V_{\text{max}}$ and $K_m$ values in the high and null UGT2B17-expressing HLM were: 27.4 and 3.8 nmol/min/mg protein, and 4.4 and 5.9 µM, respectively (Figures 3D and 3G). Overall, intrinsic clearance ($CL_{\text{int}}$) in high compared to the null UGT2B17-expressing HIM and HLM was ~1120 and 10-fold, respectively. Although UGT quantification was not the major goal of this work, we supplemented the enzyme kinetics data with proteomics information to demonstrate that only UGT2B17 protein abundance was different
across null and high UGT2B17-expressing HIM and HLM, while UGT1A9, UGT2B17, and UGT2B15 abundance was comparable (Table S7). The high and null UGT2B17-expressing HIMs and HLMs showed noticeable differences in $K_m$ values, which is likely due to involvement of UGT1A9 in the null expressors.

Fraction of DMA metabolized by UGT2B17 enzyme in intestine and liver

The estimated fraction of DMA that was glucuronidated by UGT2B17 ($f_{m,UGT2B17}$) in intestine and liver microsomes was 99.6 and 86%, respectively in the highest expressors (Figures 4E and 4F), thus suggesting a higher impact of UGT2B17-mediated metabolism in intestine compared to liver on DMA bioavailability.

Pharmacokinetic analysis of DMA and DMA-G

The area under the systemic concentration-time profile curve (AUC) of DMA-G (61.6 µmol×h/l) was ~135-fold compared to DMA (0.45 µmol×h/l), further confirming glucuronidation as the major metabolic pathway of DMA (Table 2 and Figure 4). Similarly, the $C_{max}$ of DMA-G (10.9 µmol/l) was ~135-fold as compared to DMA (0.08 µmol/l). The elimination rate constant ($K_e$) and half-lives were 0.21 h$^{-1}$ and 3.5 h, and 0.1 h$^{-1}$ and 4.7 h, for DMA and DMA-G, respectively.

DISCUSSION

DMAU is a promising oral male hormonal contraceptive, currently under clinical investigation. This study is the first to report the \textit{in vitro} and \textit{in vivo} metabolic characterization of DMA, which is the pharmacologically active metabolite of DMAU. The LC-HRMS analysis of human hepatocytes and serum samples revealed that glucuronidation is the major mechanism of DMA biotransformation. The \textit{in vitro} screening results confirmed that UGT2B17 plays a primary role in metabolizing DMA to DMA-G. UGT2B17 is a highly variable enzyme with more than 3000-fold variability in its hepatic protein abundance (Oda \textit{et al.}, 2015) with a gene deletion frequency that vary with race by ~16% in Caucasians to 96% in Asians (Xue \textit{et al.}, 2008). Therefore, we assessed the metabolism rates of DMA in the high \textit{versus} null UGT2B17-expressing HIMs and
HLMs, which showed >250- and 7-fold metabolism rates, respectively, in the high compared to null UGT2B17-expressors. This suggests that genetic polymorphism in UGT2B17 is likely leading to the variable DMA PK. Further, enzyme kinetics data was supplemented with proteomics information to demonstrate that only UGT2B17 protein abundance was different across null and high-UGT2B17 expressing HIM and HLM. The estimated $f_{m, UGT2B17}$ for intestinal and liver microsomes of the available highest expressors were 99.6 and 86%, respectively. Because UGT2B17 is mainly expressed in the intestine, it is likely that the intestine plays a major role in the first-pass elimination of DMA. Finally, human serum PK analysis showed that DMA-G AUC was >100-fold than DMA concentrations, which correlates with the extensive first-pass metabolism.

DMA is structurally similar to testosterone and retains 17β-hydroxy group involved in glucuronidation. Accordingly, our hypothesis was that DMA is a substrate of UGT2B17, which leads to its high first-pass elimination and thereby poor and variable drug disposition. In addition to UGT2B17, we also found a minor contribution of UGT1A9 in the UGT screening assay of DMA, however, UGT1A9 is poorly expressed in intestine (Basit et al., 2020; Al-Majdoub et al., 2021), which makes it less relevant to the first-pass metabolism. Further, DMA kinetics data in HLM showed 7-fold higher metabolism rates in the high compared to null UGT2B17-expressors, which confirmed that UGT2B17 is the major enzyme involved in DMA metabolism and UGT1A9 is a minor player despite of its higher hepatic abundance. Although testosterone is also metabolized by UGT2B15 (Zhang et al., 2018), the HLM data from the high versus null UGT2B17-expressing donors suggest a negligible role of UGT2B15 in DMA metabolism. Interestingly, DMA is not metabolized by other androgen-metabolizing enzymes unlike testosterone. In particular, testosterone is also metabolized by enzymes such as steroid 5 alpha-reductase 2 (5αR2), CYP3A4, sulfotransferase 2A1 (SULT2A1), and 17βHSD2 (Schiffer
et al., 2018; Zhang et al., 2018). The potential reason for this discrepancy is the absence of the C-19 methyl group in DMA (Simard et al., 2005; Attardi et al., 2008; Han et al., 2021).

Considering that DMA is primarily metabolized by UGT2B17, it can be developed as a probe substrate for in vitro as well as in vivo studies to evaluate the effect of drug-drug interactions (DDIs) and genetic polymorphisms involving UGT2B17. Further, in the high UGT2B17-expressors, the magnitude of DDI needs critical evaluation because UGT2B17 inhibition can lead to dramatic increase in the systemic exposure of substrate in these individuals. Since DMA has been shown to be relatively safe in males, it can be used as a probe at lower doses for predicting DDI potential or UGT2B17 variability in males after further validation.

The role of UGT2B17 in variable drug PK has shown to impact drug development. For example, an investigational asthma drug, MK-7246, showed 25-fold higher systemic exposure in subjects with UGT2B17 gene deletion compared to those with two gene copies (Wang et al., 2012). Additionally, UGT2B17 is involved in metabolism of anticancer agents, such as vorinostat (Wong et al., 2011) and 17-hydroxyexemestane (Chen et al., 2016). Vorinostat PK is shown to be affected by UGT2B17 CNV, with UGT2B17*2 homozygotes exhibiting serious adverse events due to reduced glucuronidation rates and increased efficacy and toxicity as compared to subjects with one wild-type allele (Wong et al., 2011). The active metabolite of exemestane, 17-hydroxyexemestane showed significantly higher (>6-fold) exposure in patients homozygous for UGT2B17 deletion compared to those with 2 copies, likely due to reduced glucuronidation (Chen et al., 2016).

Further, UGT2B17 gene deletion is a key confounder in urinary antidoping testing leading to potential false negative and positive results (Schulze et al., 2008; Kuuranne et al., 2014). The World Anti-Doping Agency screens for exogenous testosterone administration using the urinary ratio of deconjugated testosterone to epitestosterone (T/E ratio). This threshold for claiming testosterone supplementation was previously set to six in 1983 and was later reduced to four in
2005 (Mareck et al., 2010). However, baseline T/E ratio can differ by over 10-fold among different races (Jakobsson et al., 2006), and up to 20-fold between UGT2B17 deletion and high expressors (Schulze et al., 2008; Martín-Escudero et al., 2015). This also demonstrates the need of employing UGT2B17 biomarker to enable accuracy of doping tests by reducing the frequency of false negative results in the deletion subjects with low or undistinguishable changes in T/E ratio.

Since DMA is a selective UGT2B17 substrate, it will likely exhibit high inter-individual variability after oral dosing of DMAU, which can be considered for safe and cost-effective clinical trials (Figure 5). First, subjects or patients can be stratified into null-, mid- and high-expressors of UGT2B17 for less variable and potentially safer clinical trials. Although such an approach adds need for genotyping (Pereira et al., 2015), it is successfully implemented for other enzymes such as β2-adrenergic receptor (ADRB2) (Wechsler et al., 2009). Alternately, a selective UGT2B17 inhibitor can be utilized to avoid the variability from UGT2B17-mediated metabolism.

Physiologically based PK (PBPK) modeling, which accounts for the differences in the protein abundance of UGT2B17, gene frequency as well as other drug- and physiology-specific information can be used to prospectively predict the effect of UGT2B17 genetic polymorphism and CNV on the drug PK when designing clinical trials. UGT2B17-mediated DDI potential can also be predicted using such approach. Effect of enzyme inhibition by co-administered drugs as well as food, supplements, and intestinal diseases can also affect DMA metabolism due to their likely influence on intestine UGT2B17.

One of the limitations of this study was that the subjects were not genotyped for UGT2B17 due to the retrospective nature of the analysis. While we could observe inter-individual differences in the systemic profiles of DMA and DMA-G, future studies are warranted to systematically assess the effect of UGT2B17 genetic polymorphism. Moreover, we could only detect DMA-G metabolite in the human serum sample following DMAU oral administration unlike testosterone.
We could not detect androstenedione analogue of DMA in the serum samples as opposed to the hepatocyte incubations, which is likely because the androstenedione analogue is getting rapidly eliminated in sequential metabolic reactions. Nonetheless, the lack of C-19 methyl group in DMA structure provides a strong biochemical basis of the non-involvement of other enzymes that metabolize testosterone.

In summary, we characterized the *in vitro* and *in vivo* metabolism of DMA, which revealed DMA-G as the major metabolite, and a predominant role of intestinal and hepatic UGT2B17 in the metabolism. The elucidation of the DMA elimination pathways is crucial for individualized dosing and developing safer clinical trials. These findings are useful in building a top down PBPK model to understand the effect of UGT2B17 genetic polymorphism and CNV on DMA metabolism. The findings of this study can be applied for developing precision dosing and personalized medicine to achieve safe and predictable oral PK of DMAU during clinical trials.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: S.S., C.W., M.S.L., D.L.B., J.K.A., B.P.

Conducted experiments: S.S., D.A., A.B., M.L.

Performed data analysis: S.S., D.K.S, B.P.

Wrote or contributed to the writing of the manuscript: S.S., D.A., A.B., M.L., C.W., M.S.L., D.L.B., J.K.A., S.H., B.P.
References


men are strongly associated with a UDP-glucuronosyl transferase 2B17 polymorphism. J Clin Endocrinol Metab 91:687–693.


Footnotes

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The authors declare no conflict of interest.
Figure Legends

Figure 1. (A) DMAU is an ester prodrug, which is rapidly metabolized to its active metabolite, DMA. Sequential metabolism of DMA is not well characterized. (B) The mechanism of action of DMAU involves suppression of follicle stimulating hormone (FSH) and luteinizing hormone (LH) secretion from the pituitary gland leading to a cessation of spermatogenesis. (C) Chemical structures of DMA and testosterone.

Figure 2. The relative amounts of DMA (open bar) and DMA-G (filled bar) formed in human hepatocytes at (A) 1 µM and (B) 10 µM DMA concentrations at 60 and 120 min. No DMA was detected at 1 µM DMA incubation, suggesting its rapid elimination to form DMA-G. The ratios of DMA-G to DMA concentrations were ~ 2-fold at 10 µM hepatocyte incubations, confirming higher formation of the metabolite compared to the parent.

Figure 3. (A) MS response of DMA-G formation in thirteen recombinant human UGT2B17 (rhUGT2B17) enzymes. UGT1A9 and UGT2B17 were the only enzymes involved in DMA metabolism. (B) DMA metabolism kinetics in rhUGT2B17 system. (C and D) DMA-G formation kinetics in the high (red) versus null (blue) UGT2B17-expressing human intestinal microsomes (HIM; n=2 high-expressors, and n=3 low expressors) and human liver microsomes (HLM; n=3 high and null -expressors). The data points represent the mean ± standard deviation (E and F) The estimated fraction of DMA glucuronidated by UGT2B17 (f_{m,UGT2B17}) in intestine and liver microsomes was 99.6 and 86%, respectively in the high expressor. (G). Michaelis-Menten parameters and UGT2B17 protein abundance in rhUGT2B17 and the high versus null UGT2B17-expressing HIMs and HLMs, respectively.

Figure 4. Pharmacokinetic profiles of DMA-G (red circle) and DMA (blue square) following an oral administration of 100 mg DMAU. The systemic exposure (AUC) of DMA-G was >100-fold than DMA serum concentrations, confirming glucuronidation as the major mechanism of DMA disposition.
Figure 5. Potential approaches to develop safe and effective oral DMAU delivery. In the first approach, subjects can be stratified into null-, mid- and high-expressors of UGT2B17 for effective clinical trials. Second, DMA can be administered with specific UGT2B17 inhibitor to avoid the confounding effect of UGT2B17 metabolism in the first-pass elimination tissues such as intestine and liver.
Table 1. Predicted DMA metabolites assessed in human hepatocyte and serum samples after the oral administration of DMAU and their exact masses

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Exact mass [M+H]</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-DMA estradiol</td>
<td>271.1693</td>
</tr>
<tr>
<td>17-DMA estradiol-3-glucuronide</td>
<td>463.1963</td>
</tr>
<tr>
<td>17-DMA estradiol-glucuronide</td>
<td>463.1963</td>
</tr>
<tr>
<td>5α-Dihydro DMA</td>
<td>305.2475</td>
</tr>
<tr>
<td>6-OH-DMA</td>
<td>319.2268</td>
</tr>
<tr>
<td>Dihydro DMA-glucuronide</td>
<td>481.2796</td>
</tr>
<tr>
<td>DMA 3-sulfate</td>
<td>383.1887</td>
</tr>
<tr>
<td>DMA androstenedione</td>
<td>303.2319</td>
</tr>
<tr>
<td>DMA androstenedione</td>
<td>301.2162</td>
</tr>
<tr>
<td>DMA androsterone</td>
<td>305.2475</td>
</tr>
<tr>
<td>DMA androsterone-glucuronide</td>
<td>481.2796</td>
</tr>
<tr>
<td>DMA epiandrosterone</td>
<td>305.2475</td>
</tr>
<tr>
<td>DMA epiandrosterone-glucuronide</td>
<td>481.2796</td>
</tr>
<tr>
<td>DMA estrone</td>
<td>269.1536</td>
</tr>
<tr>
<td>DMA estrone-glucuronide</td>
<td>463.1963</td>
</tr>
<tr>
<td>DMA etiocholanediol</td>
<td>307.2632</td>
</tr>
<tr>
<td>DMA etiocholanolone</td>
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<tr>
<td>DMA etiocholanolone-glucuronide</td>
<td>481.2796</td>
</tr>
<tr>
<td>DMA-cysteine conjugate</td>
<td>422.2359</td>
</tr>
<tr>
<td>DMA-glucuronide</td>
<td>479.2639</td>
</tr>
<tr>
<td>DMA-glutathione conjugate</td>
<td>608.3000</td>
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</table>
Table 2. PK endpoints from the analysis of DMA and DMA-G samples following the oral administration of 100 mg DMAU

<table>
<thead>
<tr>
<th>PK endpoints (Mean ± SD)</th>
<th>DMA</th>
<th>DMA-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{AUC}_{0-24}$ (µmol/l/h)</td>
<td>0.45 ± 0.43</td>
<td>61.6 ± 18.6</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µmol/l)</td>
<td>0.08 ± 0.08</td>
<td>10.9 ± 1.9</td>
</tr>
<tr>
<td>Elimination rate constant ($K_e$) (h$^{-1}$)</td>
<td>0.21 ± 0.07</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>6 ± 2</td>
<td>4.7 ± 3.1</td>
</tr>
<tr>
<td>$T_{\text{half}}$ (h)</td>
<td>3.5 ± 0.98</td>
<td>7 ± 1.3</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.

Part A: Effect of 1 μM DMA on relative intensity over time. The relative intensity decreases significantly from 60 min to 120 min.

Part B: Effect of 10 μM DMA on relative intensity over time. The relative intensity is lower compared to DMA-G treatment, with a slight increase from 60 min to 120 min.
Figure 3.
Figure 4.
**Figure 5.**

**Approach 1: UGT2B17 Biomarker or Genotype-informed Clinical Trials**

- Population
- PBPK Modeling
- Group 1: Poor Metabolizer
- Group 2: Intermediate Metabolizer
- Group 3: Extensive Metabolizer

**Approach 2: DMA plus UGT2B17 inhibitor**

- Intestine
- Liver
- Bioavailable drug
- Unabsorbed fraction
- Metabolism