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Discovery, Biosynthesis, and Structure Elucidation of Metabolites of a Doping Agent and a Direct Analogue, Tetrahydrogestrinone and Gestrinone, Using Human Hepatocytes

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Tetrahydrogestrinone (18a-homo-pregna-4,9,11-trien-17β-ol-3-one, THG) is an anabolic androgenic steroid sold to athletes as an undetectable performance enhancer. Being an unapproved substance, no legitimate in vivo human excretion studies could be performed to identify urinary markers of this doping agent. In vitro systems were used as an alternative approach to study the human metabolism of THG and the gestrinone analogue, which is a marketed drug. Incubations of both compounds in the presence of human hepatocytes led to formation of oxidative and glucuroconjugated metabolites. Microgram quantities of the major in vitro metabolites were biosynthesized using human hepatocytes, characterized by HPLC/MS/MS, and their structures elucidated by NMR. Due to high structure similarity, both THG and gestrinone had an analogous in vitro metabolic pathway leading to successive addition of a hydroxyl and a β-glucuronic acid at C-18. This in vitro metabolite of gestrinone was consistent with a previously reported major but unknown human urinary metabolite. The structure of another metabolite of THG was proposed to be a glucuroconjugate of an oxidative product with a hydroxyl group most likely at C-16ϵ. In vitro information reported therein could significantly impact the identification of new urinary markers of THG for doping control purposes.

In June 2003, the structure of a new anabolic androgenic steroid (AAS) extracted from a spent syringe was elucidated as tetrahydrogestrinone (18a-homo-pregna-4,9,11-trien-17β-ol-3-one, THG; Figure 1) by the UCLA Olympic Analytical Laboratory.[1] Allegedly designed by Balco laboratories in the United States, THG was sold as an undetectable performance enhancing substance to elite amateur and professional athletes.[2,3] It is a direct analogue of gestrinone (Figure 1), a marketed drug for the treatment of endometriosis and banned by the world antidoping agency (WADA).[4] THG was shown to be a potent androgen and progestin[5] and was prohibited by doping control authorities.[4] THG is not approved as a drug or dietary supplement by the Food and Drug Administration (FDA), and therefore, no legitimate in vivo metabolism studies in human can be conducted for the identification of potential urinary markers for doping control purposes.[6] The only excretion study available was performed in one baboon and led to identification of THG in urine. No information was provided regarding the urinary excretion of THG metabolites in the primate.[1]

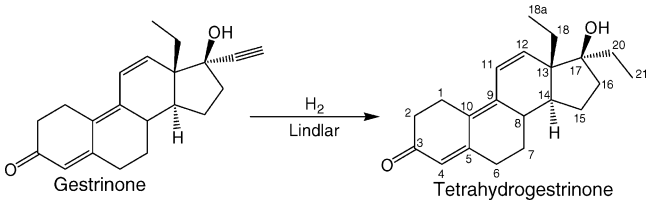

Figure 1. Chemical synthesis of THG.

It has been well documented that the pharmaceutical industry uses in vitro systems such as hepatocytes to study the metabolism of discovery compounds and ultimately postulate the main biotransformations that could occur in vivo in humans.[7] In vitro metabolism studies offer an important advantage over clinical studies in that they minimize safety issues by avoiding human in vivo exposure to potentially harmful substances. It has been previously reported that metabolites formed in human hepatocyte incubations of banned AAS (e.g., androstenedione and norandrostenedione) correlated well with urinary metabolic profiles observed in humans following *per os* administration.[8] Furthermore, in vitro metabolic systems can be used to produce sufficient amount of metabolites for structure elucidation by nuclear magnetic resonance (NMR).[9−11] These can then be used as

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authentic standards for in vivo sample analysis and biological activity testing. Such an approach has been used to identify new in vitro metabolites of norandrostenedione,[12] a precursor of nortestosterone banned by the WADA.[4]

This paper reports the use of in vitro systems as an alternative approach to in vivo clinical studies in humans, to determine the metabolism of THG and gestrinone. Both doping agents were incubated in the presence of human hepatocytes from five different donors in order to evaluate their metabolic profiles. Subsequent biosynthesis, isolation, and characterization by high-pressure liquid chromatography (HPLC) coupled to photodiode array (PDA), fluorescence, mass spectrometer (MS) or MS/MS detectors, and NMR were done to elucidate the structure of the major metabolites. To evaluate the in vivo relevance of these new metabolites of THG, in vitro versus in vivo correlation was assessed for gestrinone based on urinary metabolites previously reported in the literature in humans following *per os* administration.[13]

EXPERIMENTAL SECTION

Reagents. Gestrinone was obtained from APIN Chemicals (Oxon, U.K.). β-Glucuronidase from *Escherichia coli* type IX-A, NADPH, and 99% absolute ethanol were purchased from Sigma (St. Louis, MO). Lindlar catalyst (5% palladium on calcium carbonate poisoned with lead) was purchased from Strem Chemicals (Newburyport, MA). Formic acid and monobasic sodium phosphate were obtained from Anachemia (Montreal, Canada), and ammonium hydroxide was obtained from A&C American Chemicals (Saint-Laurent, Canada). Methanol and acetonitrile were HPLC grade and obtained from Fisher (Nepean, Canada).

THG Synthesis and Purification. THG was synthesized by reduction of the alkyne portion of gestrinone to an alkane (Figure 1). To a solution of gestrinone (50 mg, 0.162 mmol) in EtOH (2 mL, 0.8 M) at room temperature was added Lindlar catalyst (5% palladium on calcium carbonate poisoned with lead) (10 mg) and a balloon of hydrogen (gas). The mixture was stirred at room temperature for 30 min. The reaction was stopped, filtered through Celite, and washed with 20 mL of ethanol. Purification was achieved by preparative HPLC using a Waters 600 Multisolvent delivery system with a Waters 486 tunable absorbance detector (Milford, MA) and an XTerra C_{18} 5 μm, 30 × 100 mm column (Waters) using 45% acetonitrile in water at a flow rate of 12 mL/min for 30 min. The injection volume was 250 μL, and the single-wavelength UV detector was set at 345 nm. Fractions were collected manually, analyzed by HPLC/PDA/fluorescence to determine purity. Pure fractions were then pooled and evaporated to dryness. Scale-up to 150 mg of gestrinone and 30 mg of Lindlar catalyst was performed using conditions previously described. Purity and structure of THG were confirmed by HPLC/PDA/MS and NMR analysis.

In Vitro Metabolism. (1) Human Hepatocyte Isolation and Incubation. As approved by the bioethic committee of the Centre Hospitalier de l'Université de Montréal (CHUM), human liver tissues were obtained from fully informed consenting patients (age (gender): 64 (M), 72 (F), 72 (F), 56 (F), 72 (F)) undergoing partial hepatectomies at a local hospital due to hepatic metastasis of adenocarcinoma. Tissue samples were transported from the operating room in ice cold University of Wisconsin solution, and human hepatocytes were isolated as previously described.[14,15] In summary, a two-step collagenase perfusion was performed and the viability of the cells was determined by tryptan blue uptake. Preparations showing cell viability under 80% were discarded.

For small-scale incubations, 0.5 mL of 2×10^6 cells/mL in Krebs−Henseleit buffer (pH 7.4, with 3 g/L Hepes added) (Sigma) were preincubated for 20 min at 37 °C under 95%:5% O_2/CO_2 (BOC Gases; Montreal, Canada) in a 48-well plate. A final concentration of 50 μM gestrinone or THG (2.5 μL of a 10 mM stock solution in acetonitrile) was added to each well and incubated at 37 °C under 95%:5% O_2/CO_2 for 2 h. The incubations were quenched by adding one volume of acetonitrile to each well. Control incubations included quenched incubates spiked with the parent and a blank incubation. The quenched samples were transferred into Eppendorf tubes, vortexed, and centrifuged at 14 000 rpm for 10 min.

For large-scale incubations, a protocol similar to that described above was used except that a total volume of 75 mL of 2×10^6 cells/mL solution was divided into two 125-mL sterile plastic Erlenmeyer flasks and 50 μM gestrinone or THG (dissolved in acetonitrile as above) was added to each solution after a 20-min preincubation period. After 3-h incubation, the solution was transferred into separate tubes and frozen at −80°C pending metabolite isolation.

(2) β-Glucuronidase Hydrolysis. Following solid-phase extraction isolation of desired phase II metabolites using the protocol described in under Solid-Phase Extration and methanol evaporation, 500 μL of 125 mM phosphate buffer (pH 6.9) and 2000 units of β-glucuronidase were added to the dried fractions. Control incubations were also conducted in the absence of β-glucuronidase along with a blank sample. All samples were incubated for 1 h at 50 °C in a water bath. Samples were quenched with one volume of acetonitrile, vortexed, and centrifuged for 10 min at 14 000 rpm.

(3) Human Recombinant P-450 Incubations. The incubations were performed using 50 pmol/mL human recombinant P450 enzyme (rP450) in 125 mM phosphate buffer (pH 7.4), with 1 mM NADPH and 20 μM gestrinone or THG added, in a final volume of 500 μL. The rP450 (Supersomes) used were purchased from BD Gentest Corp. (Bedford, MA) and included the following: 1A1+OR, 1A2+OR, 1B1+OR, 2A6+OR+b5, 2B6+OR+b5, 2C8+OR, 2C9*1+OR+b5, 2C9*2+OR, 2C9*3+OR, 2C18+OR, 2C19+OR+b5, 2D6*1+OR, 2E1+OR+b5, 2J2+OR+b5, 3A4+OR+b5, 3A5+OR, 3A7+OR+b5, 4A11+OR, 4F2+OR+b5, 4F3A+OR+b5, 4F3B+OR+b5, 4F12+OR+b5, and an insect cell control. The samples were incubated in a water bath at 37 °C for 1 h. Samples were quenched with one volume of acetonitrile, vortexed, and centrifuged for 10 min at 14 000 rpm.

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Metabolite Isolation. (1) Solid-Phase Extraction. The protocol used here was adapted from a reported procedure for selective isolation of conjugated and unconjugated metabolites using anionic solid-phase extraction (SPE) cartridges.[16] The incubation mixtures were thawed at room temperature and diluted 1:1 with 0.1% formic acid$_{(aq)}$. Samples were then vortexed and centrifuged for 10 min at 4300 rpm. The supernatant obtained was loaded on a conditioned Oasis MAX 6 mL SPE cartridge (Waters) and then washed once with 3 mL of 2% $NH_4OH_{(aq)}$ and three times with 3 mL of methanol to recover unconjugated metabolites. The glucuroconjugated metabolites were eluted in six 3-mL fractions of 2% formic acid in methanol. All washings and eluates were collected for Analytical HPLC/PDA/Fluorescence Analysis. The fractions containing metabolites of interest were pooled for further purification by preparative HPLC.

(2) Preparative HPLC. Methanolic fractions obtained from SPE isolation were evaporated to dryness and reconstituted in 425 μL of a 1:1 mixture of methanol and water. Isolation was carried out using a Waters 2790 HPLC system and Waters 996 PDA detector. An injection volume of 100 μL, a flow rate of 1 mL/min, and an XTerra MS C_{18} 2.5 μm, 4.6 × 50 mm column (Waters) were used. The mobile phase was composed of 0.1% formic acid $_{(aq)}$ (eluent A) and methanol (eluent B). An isocratic elution, followed by a quick gradient, was used to separate the metabolites; 50% eluent B for 17 min, increase to 90% eluent B over 1 min, and remain at 90% eluent B for 4 min. All peaks were collected manually and analyzed by HPLC/PDA/fluorescence for purity determination. Pure fractions were combined and evaporated to dryness prior to metabolite characterization.

In Vitro Metabolite Characterization. (1) Analytical HPLC/PDA/Fluorescence Analysis. An Agilent (Palo Alto, CA) 1100 Series liquid chromatograph equipped with a PDA and a fluorescence detector was used with a Zorbax RX-C_{18}, 5 μm, 4.6 × 150 mm column (Agilent). The mobile phase was composed of 0.1% formic acid $_{(aq)}$ (eluent A) and acetonitrile (eluent B). The gradient elution program was from 10 to 60% eluent B over 17 min, to 90% eluent B in 1 min, and then isocratic at 90% eluent B for 4 min. Prior to HPLC analysis, supernatants obtained from in vitro incubates were diluted 1:1 with water to decrease the organic content to less than 25%. An injection volume of 50 μL, a flow rate of 1 mL/min, and a column temperature of 40 °C were used. The PDA detector was set to acquire from 210 to 400 nm. The fluorescence detector was programmed with an excitation wavelength at 365 nm and an emission wavelength at 465 nm. The fluorescence detector was also programmed to perform a multiple-wavelength emission scan (λ_{exc} at 365 nm; λ_{em} 390−500 nm, in 5-nm steps) or a multiple-wavelength excitation scan (λ_{exc} 240−440 nm, in 5-nm steps; λ_{em} at 465 nm).

(2) HPLC/PDA/MS and HPLC/PDA/MS/MS Analysis. A Waters 2790 chromatograph and a Waters 996 PDA were coupled with a Micromass ZQ mass spectrometer (Manchester, England). HPLC condition used were identical to those reported. The mass spectrometer was equipped with an electrospray ionization source and was operated in both positive and negative ion modes with a cone voltage of 30 V. The masses scanned in both ion modes were from 100 to 800 u.

For the HPLC/PDA/MS/MS, a Waters 2790 chromatograph and a Waters 996 PDA were coupled with a Micromass Quattro LC mass spectrometer. The same parameters for the liquid chromatography were used as for HPLC/PDA/fluorescence analysis. The mass spectrometer was equipped with an electrospray ionization source and was operated in positive ion mode. The cone voltage was set at 30 V, and daughter ion scans were performed using collision energies of 10, 20, or 30 eV.

(3) NMR Analysis. All NMR spectra were acquired on a Varian (Palo Alto, CA) Inova 600-MHz spectrometer equipped with an inverse H/C/N 5-mm cryogenic probe. Gestrinone and THG were dissolved in 160 μL of acetonitrile-d_3 and placed in a standard 3-mm NMR tube. Metabolites were prepared by first dissolving in 115 μL of DMSO-d_6 (Cambridge Isotopes) to which was added 5 μL of D_2O (Cambridge Isotopes). The solution was placed in a 3-mm Shigemi (Shigemi Inc.) NMR tube matched to DMSO-d_6. Proton and carbon assignments were made using a combination of 1D−^{1}H spectra and 2D gCOSY, NOESY, HSQC, and HMBC experiments.

RESULTS AND DISCUSSION

Gestrinone, a commercially available compound, was used as a starting material for the organic synthesis of THG. In the presence of Lindlar catalyst, the ethynyl moiety of gestrinone was reduced to an ethyl group (Figure 1). Due to the presence of three conjugated double bonds and a ketone moiety, both gestrinone and THG displayed a UV spectrum with λ_{max} at 345 nm and could be monitored by fluorescence detection at λ_{exc} 365 nm to λ_{em} 465 nm. UV and fluorescence spectra of THG are presented in Figure S-1 (Supporting Information). When the reaction was allowed to go to completion, THG was formed, as well as several overreduced side products devoid of a fluorescence signal (λ_{exc} at 365 nm to λ_{em} at 465 nm) with λ_{max} at 308 or 248 nm instead of 345 nm. By HPLC/MS, the corresponding $[M + H]^+$ ions of these side products were 6 and 8 u higher than that of gestrinone ($[M + H]^+$ at m/z 309), respectively. In addition, totally reduced compounds with $[M + H]^+$ at m/z 319 and showing no UV signal were also formed. Hence, successive reduction of the ethynyl group and of the alkene moieties at C-11, C-9, and C-4 of gestrinone occurs under these conditions, leading to formation of numerous undesirable overreduced products. Since these were not resolved from THG by preparative HPLC, the reaction was repeated under the same conditions but the reaction was stopped once total consumption of gestrinone and a 1:1 ratio of dihydrogestrinone to THG had been achieved. No side products were observed under these conditions, and THG was purified by preparative HPLC. The reaction was scaled up to 150 mg of gestrinone and 30 mg of Lindlar catalyst and yielded 30.1 mg of THG (>99% pure). The structure of THG was confirmed by HPLC/PDA/MS/MS and NMR analysis and results for THG, as well as for gestrinone, are presented in Figures S-2 and S-3 (Supporting Information) and Table 1.

The liver is known to be an important site of metabolism for a large variety of xenobiotics. Considering its potential implication in the in vivo metabolism of THG and gestrinone, human hepatocytes from five different donors were isolated and incubated in the presence of both compounds, separately. Quenched incubations were analyzed by HPLC/PDA/fluorescence and HPLC/PDA/MS, and metabolic profiles obtained for THG are

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Table 1. ^{1}H and ^{13}C Chemical Shifts for THG, M1, and M2 as Well as for Gestrinone and Md

	THG		THG − M1	THG − M2		gestrinone		gestrinone − Md	
position	^{1}H	^{13}C	^{1}H (partial)	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	2.89, 2.81	24.05	2.78, 2.68	2.85, 2.60	24.05	2.90, 2.84	24.19	2.88, 2.64	24.18
2	2.39	36.41	2.30	2.33, 2.28	36.75	2.40	36.48	2.34, 2.30	36.83
3		198.16		198.5			198.14		198.29
4	5.70	122.88	5.64	5.63	123.26	5.72	123.19	5.65	123.49
5		156.53			156.87		156.3		156.51
6	2.60	31.09	2.49	2.54	31.19	2.61	31.08	2.57	31.24
7	1.91, 1.25	2.01	1.80, 1.12	1.84, 1.09	27.59	1.92, 1.28	27.08	1.85, 1.08	27.58
8	2.59	37.52	N/O	2.72	37.35	2.58	37.5	2.73	37.33
9		142.26			142.72		141.46		142.44
10		126.57			127.45		127.22		127.68
11	6.64	124.34	6.56	6.55	125.12	6.69	124.57	6.59	125.10
12	6.43	141.51	6.33	6.33	141.23	6.50	140.19	6.41	140.60
13		50.91			55.03		51.6		55.27
14	1.73	49.02	N/O	1.60	49.83	2.02	49.14	1.98	49.47
15	1.59, 1.50	22.25	2.16, 1.33	1.85, 1.38	23.69	1.73, 1.52	22.02	1.90, 1.48	23.01
16	2.00, 1.64	33.39	3.80	1.87, 1.74	36.77	2.30, 2.10	39.35	2.29, 2.13	41.53
17		83.47			83.57		78.12		78.46
20	1.60, 1.40	30.19	1.47, 1.06	1.58, 1.16	31.53		88.56		90.11
21	0.95	7.01	0.80	0.85	7.93	2.74	72.64	3.22	N/O
18	1.81, 1.47	24.13	1.66, 1.28	4.03	80.52	1.72, 1.48	22.59	3.94	80.00
18a	1.02	10.75	0.89	1.38	21.13	1.01	10.49	1.39	20.62
1′			4.12	4.27	103.74			4.21	104.10
2′			2.96	2.91	74.19			2.98	74.18
3′			3.10	3.10	77.11			3.08	77.30
4′			3.05	3.02	72.45			2.99	72.50
5′			3.12	3.17	74.1			3.13	74.01
6′					172.64				172.41

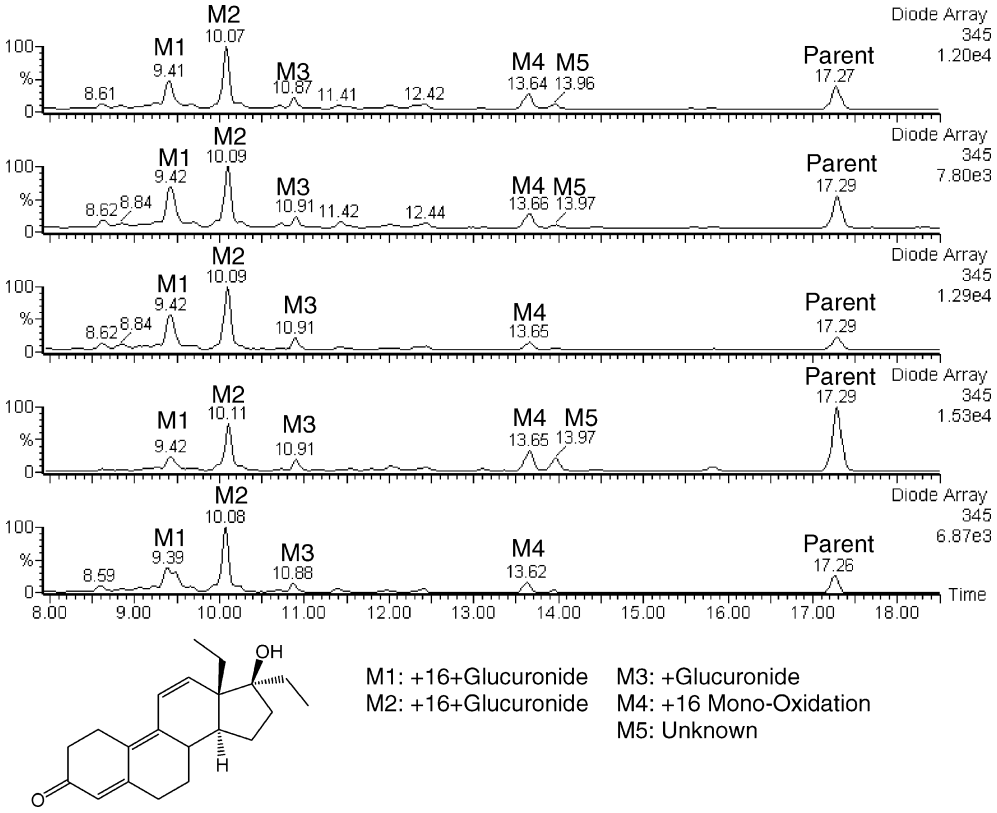

Figure 2. Chromatograms of fresh human hepatocyte incubations (n = 5 donors) of THG generated by HPLC/PDA (at λ 345 nm).

pictured at Figure 2. The percent THG remaining after a typical 2-h incubation ranged from 6 to 26% with an average of 13%. All metabolites observed by UV and MS displayed λ_{max} at ~345 nm and a fluorescence signal, indicating no reduction of the conjugated system of THG during the incubation. Oxidation and glucuronidation were the main biotransformations observed based on HPLC/MS analysis. In ESI positive ion mode, both M1 and M2 displayed $[M + H]^+$ ions at m/z 505, 192 u higher than that of the parent drug, consistent with a combination of oxidation (+16 u) and glucuronidation (+176 u). In the case of M3, the [M +

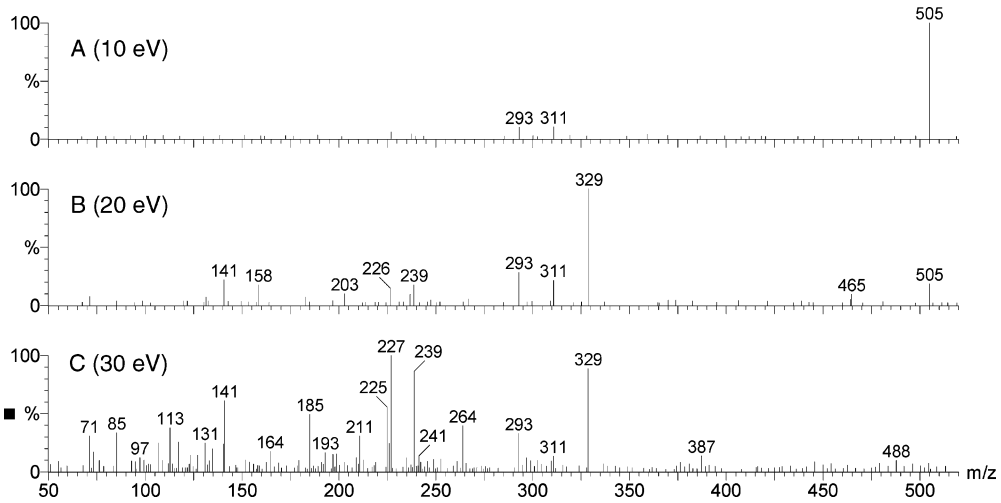

Figure 3. HPLC/MS/MS of M1 of THG generated in ESI+ ion mode at (A) 10, (B) 20, and (C) 30 eV collision energy (daughter ions of $[M + H]^+$ at *m*/*z* 505).

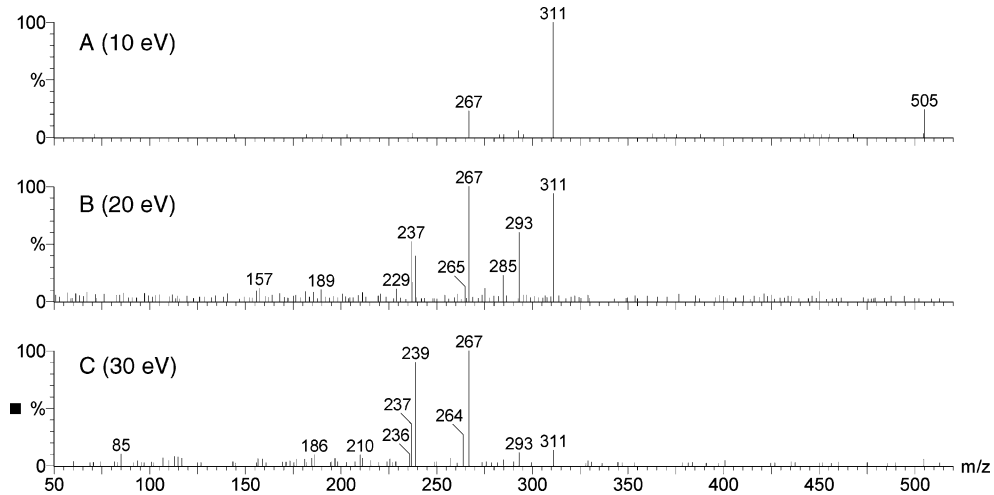

Figure 4. HPLC/MS/MS of M2 of THG generated in ESI+ ion mode at (A) 10, (B) 20, and (C) 30 eV collision energy (daughter ions of $[M + H]^+$ at *m*/*z* 505).

$H]^+$ ion was 176 u higher than that of THG, suggesting the presence of a glucuroconjugate, whereas for M4, the $[M + H]^+$ ion at m/z 325 was indicative of a monooxidation. An unknown metabolite, M5, was also observed in some incubations. No sulfate adducts of the parent or oxidative metabolites were observed in ESI− ion mode in any incubation analyzed. Interestingly, the metabolic profiles of THG obtained using fresh human hepatocytes from five different donors were very similar, leading overall to the formation of M1 and M2 as the main metabolites. To characterize and elucidate the structure of these two metabolites by NMR, larger quantities were required.

A large-scale biosynthesis was performed using fresh human hepatocytes from donor 4. Under the incubation conditions used for the large scale (3 h) reaction, complete consumption of THG was noticed, leading to formation of 18% M1 and 33% M2, based on total peak area (λ at 345 nm). An anionic SPE cartridge was used to selectively isolate glucuroconjugated from unconjugated steroids. Then, M1 and M2 from the glucuroconjugated fraction were isolated by preparative HPLC. Based on NMR analysis, approximately 15 and 60 μg of M1 and M2 were isolated, respectively.

Isolated THG metabolites were then characterized by HPLC/MS/MS and NMR to elucidate their structures. HPLC/MS/MS analysis was performed on M1 and M2 at collision energies of 10, 20, and 30 eV and daughter ions of $[M + H]^+$ at m/z 505 were acquired (Figures 3 and 4). In general, M2 showed significantly more fragmentation than M1 under the same MS/MS conditions. At a collision energy of 10 eV, a parent ion at m/z 505 was largely predominant for M1 whereas for M2, the ion at m/z 311 $[M - \text{glucuronide} - H_2O]^+$ was the main species present. As the collision energy increased, different fragmentation patterns were observed. For M1, the neutral loss of the glucuronic acid (176 u) to m/z 329 was preponderant, even at 30 eV. In the case of M2, sequential glucuronic acid and water losses was observed at 20 eV (to m/z 311 and 293), and the formation of fragments at m/z 267 and 239 was favored at higher collision energy (30 eV). Based on their dissimilar fragmentation patterns observed by MS/MS, it was speculated that the sites of hydrolylation or glucuronidation on M1 and M2 were different.

Complete proton and carbon NMR assignments were performed on THG, M1, and M2 and are shown in Table 1. In the case of THG, two triplets each integrating for three protons were

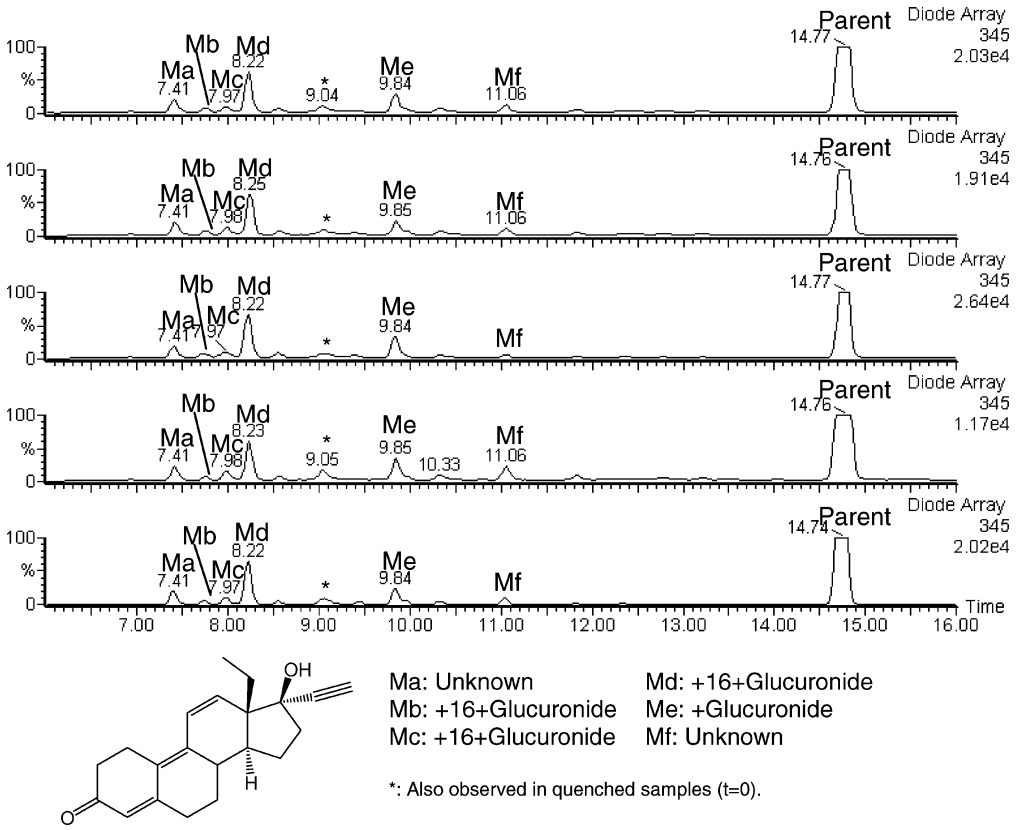

Figure 5. Chromatograms of fresh human hepatocyte incubations (n = 5 donors) of gestrinone generated by HPLC/PDA (at λ 345 nm).

observed, corresponding to the methyl protons at C-18a and C-21. In the case of M2, one of the two triplets was replaced with a doublet that coupled to a quartet integrating for one proton at 4.0 ppm, suggesting that an oxidation had occurred at either position 18 or 20. The triplet correlated to the readily assignable carbon at position 17 in the HMBC experiment identifying carbon 18 as the site of oxidation in M2. In addition to the expected signals for the steroid, signals for the glucuronic acid were also observed. The anomeric proton had a $^3J_{HH}$ coupling constant of 7 Hz, which is consistent with it being in the β configuration. To determine through which of the two possible hydroxyl groups the β-glucuronic acid was linked to the steroid, the HMBC spectrum was examined. A strong correlation was observed between the anomeric proton at C-1′ of the β-glucuronic acid and C-18 of M2. A similar interaction was also observed between the proton at C-18 and the C-1′ carbon of the glucuronide. This unambiguously confirmed that the β-glucuronic acid was attached to M2 via its newly introduced hydroxyl function at C-18 (structure shown in Figure 7).

In the case of M1, the small amount of metabolite that was isolated limited the NMR experiments that could be acquired. In the proton spectrum, two triplets integrating for three protons each, corresponding to the methyl groups, were observed. This implied that the two ethyl groups of M1 were unmodified and suggested that the oxidation occurred on one of the steroid rings. Using the 1D ^{1}H NMR spectrum and the 2D-gCOSY data, all protons excluding those at C-14, C-15, and C-16 were determined to be unchanged. Observation of an alcoholic methane proton at 3.8 ppm excludes C-14 as the site of hydroxylation. Although partially obscured, a CHOH−CH_2 proton coupling pattern was present, indicating that the site of hydroxylation was either C-15 or C-16. Typical ^{1}H NMR signals for a β-glucuronide were observed in a 1:1 stoichiometric ratio with those of the steroid core. However, insufficient material was isolated to successfully acquire the HMBC spectrum that would allow the identification of the glucuronide linkage site and confirm that the newly introduced alcohol was at C-15 or C-16. It is well known for AAS that C-16 is an important site of hydroxylation in human, whereas oxidation at C-15 has never been reported in vivo in human.[17] It was therefore proposed that hydroxylation was likely to occur at C-16ϵ. The site of glucuronidation would be on either of the alcohol moieties at C-16ϵ or C-17β. A tentative structure is shown in Figure 7.

Gestrinone was incubated side by side with THG in human hepatocytes from the same five donors in order to compare their metabolic profiles (Figure 5). Similar profiles were obtained across donors with, on average, 68% gestrinone remaining after the 2-h incubation. Six potential metabolites were identified in the supernatant of the incubations by HPLC/PDA/MS. Two metabolites, Ma and Mf, remain unknown as their mass spectra did not show diagnostic mass changes as compared to the parent compound. Mb, Mc, and Md were all hydroxylated (+16 u) and glucuroconjugated (+176 u) metabolites with a parent ion at m/z 501 in ESI+. The parent ion for Me was determined to be at m/z 485, which is in agreement with a glucuronide adduct of gestrinone. As for THG, a large-scale biosynthesis was performed using cells from donor 4, and solid-phase extraction procedures were used to selectively isolate glucuroconjugated from unconjugated

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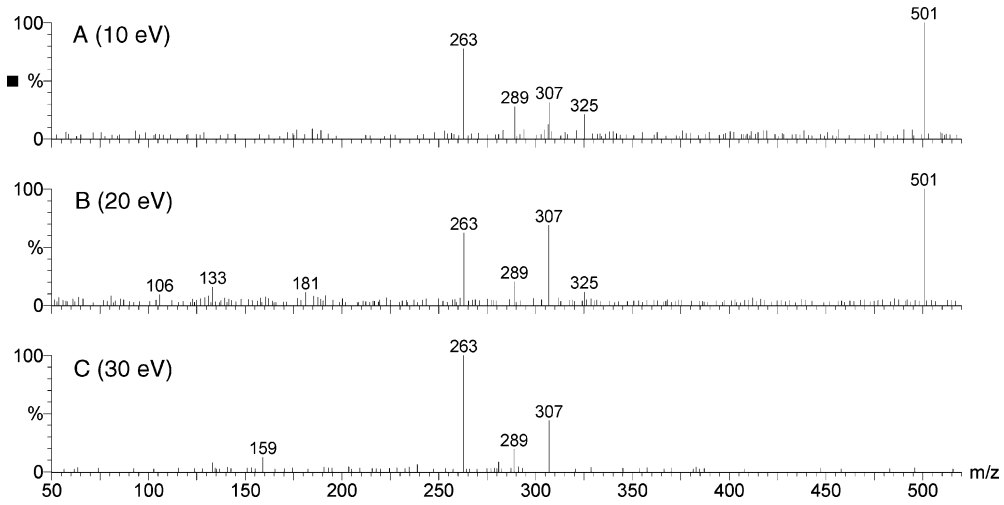

Figure 6. HPLC/MS/MS of the main metabolite of gestrinone (Md) generated in ESI+ ion mode at (A) 10, (B) 20, and (C) 30 eV collision energy (daughter ions of $[M + H]^+$ *at m/z* 501).

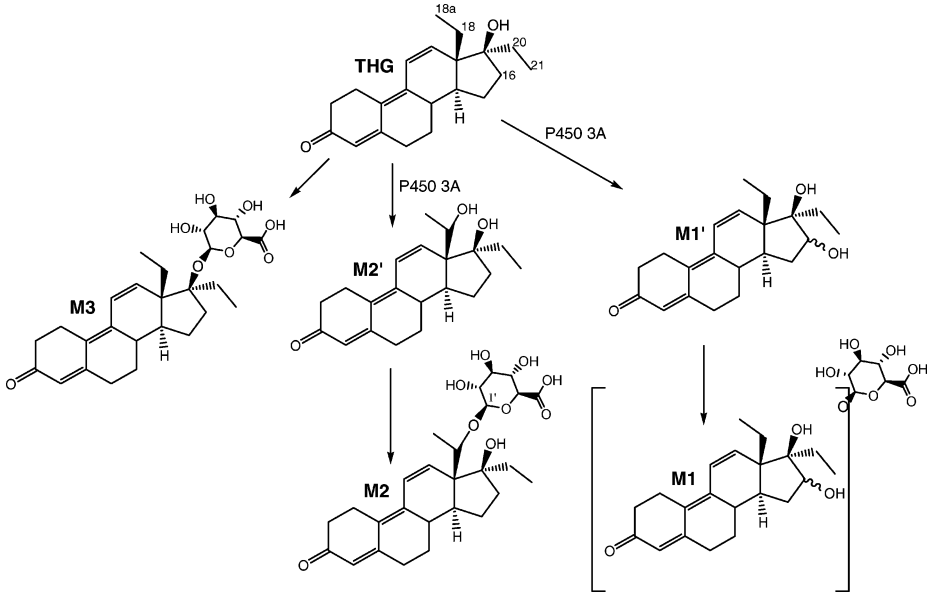

Figure 7. Proposed metabolic pathways leading to formation of main in vitro metabolites of THG in fresh human hepatocytes.

metabolites. All major metabolites were isolated by preparative HPLC, although only the major metabolite (Md) gave sufficient amount to allow NMR characterization.

HPLC/MS/MS analysis of Md revealed a fragmentation pattern similar to that seen for M2 of THG. HPLC/MS/MS analysis was performed on Md at collision energies of 10, 20, and 30 eV, and daughter ions of $[M + H]^+$ at *m/z* 501 were acquired (Figure 6). Main ions observed were at *m/z* 501 $[M + H]^+$, 307 $[M - \text{glucuronide} - H_2O]^+$, *m/z* 289 $[M - \text{glucuronide} - 2H_2O]^+$, and 263. All major fragments of Md were 4 u lower than those of M2 of THG, most likely due to the presence of an alkyne moiety at C-20 and C-21. Based on these data, it was suggested that Md could be a direct analogue of the M2 with hydroxyl and glucuronide moieties linked to C-18.

Both gestrinone and its metabolite (Md) were characterized by NMR and chemical shifts (^1H and ^{13}C) are presented at Table 1. The 1D ^1H NMR spectrum of gestrinone had one triplet integrating for three protons that represented the protons at C-18a. This triplet was no longer present in the 1D ^1H NMR of Md, and a doublet integrating for three protons was located upfield at 1.4 ppm. There was also a signal integrating for a single proton at 3.9 ppm from a hydrogen located on a carbon linked to an oxygen. This suggested that the site of hydroxylation was at C-18, analogous to that seen in M2. A strong correlation was observed between the anomeric proton at C-1′ of the β-glucuronic acid and C-18 of Md. A similar interaction was also observed between the proton at C-18 and the C-1′ carbon of the glucuronide. This confirmed that the β-glucuronic acid was attached to Md via its newly introduced hydroxyl function at C-18 (Figure 8).

M1, M2 (THG), and Md (gestrinone) are all oxidative metabolites conjugated to a glucuronic acid. As depicted at Figures 7 and 8, both M2 and Md would have a similar metabolic pathway with first an oxidation at C-18, followed by addition of a β-glucuronic acid on the new alcohol functionality at C-18. In the case of M1, a combination of hydroxylation, most probably at C-16ϵ, and glucuronidation would be implicated in its formation.

To evaluate whether the oxidative intermediates leading to the formation of glucuronide adducts were present in human hepa-

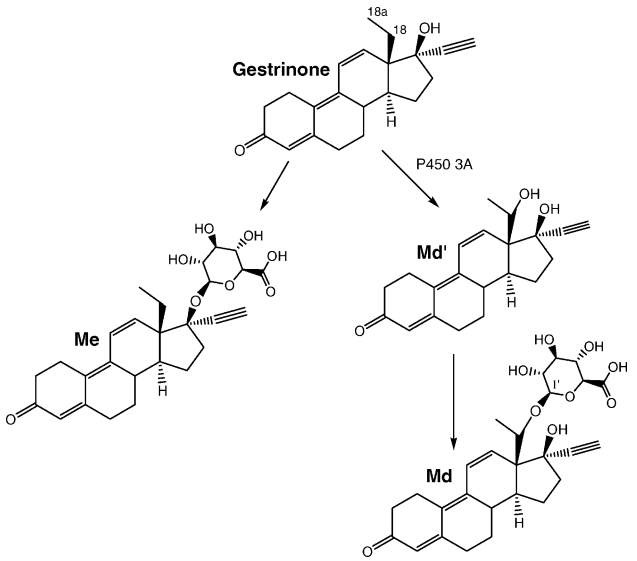

Figure 8. Proposed metabolic pathways leading to formation of main in vitro metabolites of gestrinone in fresh human hepatocytes.

tocyte incubations, isolated metabolites were treated with β-glucuronidase to hydrolyze M1, M2, and Md to their unconjugated forms (M1′, M2′, and Md′). None of the aglycon metabolites were detected in the human hepatocyte incubations of THG and gestrinone, suggesting a fast and efficient glucuronidation of oxidative metabolites with hydroxyl moieties at C-18 or C16ϵ in human hepatocytes. By HPLC/MS/MS, the main fragments of Md′ and M2′ (analogous metabolites with an alcohol at C-18) were 62 u lower than the parent $[M + H]^+$ (from m/z 325 to 263 for Md′ and from m/z 329 to 267 for M2′) (Figure S-4 Supporting Information). Minimal fragmentation could be observed for these unconjugated metabolites, as compared to their conjugated analogues. It may be possible that the presence of glucuronide adducts would affect their fragmentation pattern. Further work would be needed to elucidate the mechanism implicated in the formation of ions at m/z 263 or 267 for Md′ and M2′, respectively. In the case of M1′, its fragmentation pattern was similar that of M1, with the exception of the neutral glucuronic acid loss. In the presence of β-glucuronidase enzyme, glucuroconjugates of THG (M4) and gestrinone (Me) were converted back to parent compounds. This confirmed that glucuronidation, most probably on the hydroxyl at C-17β, would be another biotransformation implicated in the in vitro metabolism of THG and gestrinone (Figures 7 and 8).

P450s are known to play an important role in the metabolism of many xenobiotics in human. Considering that a primary route of metabolism of THG and gestrinone involved an oxidative process, incubations in microsomes expressing mainly P450 enzymes were conducted. Formation of Md′ (for gestrinone), M1′, and M2′ (for THG) were observed in the presence of rP450 3A4, and to a lesser extent with rP450 3A5 and 3A7. Since P450 3A is the most abundant subfamily in human liver, it is likely to be an important player in the in vivo metabolism of THG and gestrinone.[18]

Major biotransformations reported for most AAS are usually hydroxylation at C-2, C-6, or C-16, oxidoreduction of alcohol or ketone at C-3 and C-17, reduction of the C-4−5-ene function, and phase II conjugation (glucuronidation and sulfation).[17] Structures of THG and gestrinone are unique due to the presence of four conjugated double bonds in A, B, and C rings, leading to highly planar molecules with 3D structures distinct from known AAS (e.g., testosterone). Only trenbolone (estra-4,9,11-trien-17β-ol-3-one; Figure S-5 Supporting Information), a veterinary product banned by the WADA, shows structural similarities with its conjugated system. However, trenbolone is devoid of ethyl or ethynyl side chains at C-13 and C-17. When it was administered to human, no metabolism was reported on the A, B, and C rings.[19] Main metabolites excreted in urine were formed via isomerization at the secondary C-17 (D ring) and glucuronidation. Steroids with ethyl, ethylnyl, or both side chains attached to C-13 or C-17 but with saturated B and C cycles have been studied in vivo in humans (structures shown at Figure S-5 Supporting Information). As for THG and gestrinone, some biotransformations observed involved oxidations at C-16 (norgestrel)[20] or on ethyl side chains (ethylestrenol and norethandrolone)[21,22] at the terminal carbon. However, it is well documented that secondary carbons, such as C-18 of our two analytes, are more prone to P450-mediated oxidation than primary carbons, such as C-18a.[23−25]

When gestrinone was dosed orally in human, parent and urinary metabolites were observed in the hydrolyzed fraction of urine samples.[13] These were identified as glucuronide adducts by enzymatic hydrolysis using β-glucuronidase. Kim et al. have characterized unconjugated forms of two unknown urinary metabolites of gestrinone by HPLC/MS/MS using a protocol similar to what was mentioned above. Reported mass spectra for one of the in vivo metabolites were in good agreement with those generated for the unconjugated form of Md (Md′). By HPLC/MS in ESI positive ion mode, both the in vivo metabolite and Md′ have $[M + H]^+$ at m/z 325 with a major fragment at m/z 263. HPLC/MS/MS on the ion at m/z 263 led to similar fragmentation patterns with major daughter ions at m/z 235, 217, 205, 167, and 153.[13] Furthermore, the analysis of its TMS-enol TMS-ether derivative by GC/MS, reported in the literature, led to formation of fragments at m/z 423 ($[M - 117\ u]^{+\cdot}$) and 117 (e.g., $[CH(OSi(CH_3)_3)CH_3]^{+\cdot}$), characteristic for an ethyl side chain containing a TMS-ether moiety.[13] Based on these observations, the metabolite of gestrinone identified in vitro in human hepatocyte incubations (Md) could correspond to this unidentified urinary metabolite reported in the literature following a *per os* dose of gestrinone in humans. In addition, the glucuroconjugated form of gestrinone observed by Kim et al. is consistent with Me formed in human hepatocyte incubations of gestrinone. A co-injection of authentic

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standards of Md and Me and urine samples from the excretion study would be needed to further confirm this hypothesis.

Doping control laboratories currently use GC/MS to evaluate the presence of urinary markers of banned AAS. As a generic method, TMS derivatization is conducted on isolated urinary steroids prior to GC/MS analysis. Chemical derivatization methods reported in the literature were tested to synthese TMS-ether TMS-enol gestrinone.[13,26] None of them led to the formation of the desired derivative based on GC/MS analysis. The main side product formed had an $M^{+\cdot}$ at m/z 448, 4 u lower than that expected for TMS-ether TMS-enol gestrinone. Similar difficulties were reported for the synthesis and analysis of TMS-enol TMS-ether derivatives of THG and trenbolone, both containing a conjugated system.[1,19] The lack of stability encountered was most probably due to chemical or thermal degradation during the derivatization procedure or in the inlet of the GC/MS. This would have an important impact on the detection of urinary markers of THG in a doping control perspective. Therefore, detection of THG metabolites would be more suitable by HPLC using a combination of PDA, fluorescence, and MS or MS/MS detectors.

CONCLUSION

Using fresh human hepatocytes, the in vitro metabolism of THG and gestrinone was studied, and microgram quantities of the major metabolites were biosynthesized, isolated, and characterized by HPLC coupled to PDA, fluorescence, MS, and MS/MS detectors and NMR for structure elucidation. Oxidation at C-18 and most likely at C-16ϵ and glucuronidation were the main biotransformations observed. In vitro metabolism information generated on THG could have a significant impact on the understanding and the prediction of in vivo metabolism of this unapproved xenobiotic, considering that the main metabolite of gestrinone identified in vitro was consistent with a major urinary metabolite reported as unknown in human.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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