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PREPARATION AND ANALYSIS OF TETRAHYDROGESTRINONE (THG), A RECENTLY REPORTED 'DESIGNER' ANABOLIC STEROID: A PRELIMINARY REPORT

W. Karpiesiuk, A. Lehner, C. Hughes, F. Guan*, C. Uboh†, L. Soma*, C. Bratton**, L. Kellerman** and T. Tobin

*University of Kentucky, Lexington, Kentucky; *University of Pennsylvania, Kennet Square, Pennsylvania; †West Chester University, West Chester, Pennsylvania; **Neogen Corporation, Lexington, Kentucky, USA*

Abstract

Tetrahydrogestrinone (THG, 13-ethyl-17β-hydroxy-18,19-dinor-17α-pregna-4,9,11-trien-3-one, $C_{21}H_{28}O_2$, 312 mw), reportedly used by athletes due to its anabolic properties, is a synthetic 19-norsteroid closely related to gestrinone (13-ethyl-17β-hydroxy-18,19-dinor-17α-pregna-4,9,11-trien-20-yn-3-one, $C_{21}H_{24}O_2$, 308 mw), originally developed as an oral contraceptive for women. Recent reports detail the use of THG by athletes to enhance athletic performance, and the FDA has banned THG as a 'designer drug'. THG has been considered difficult to analyse because of its instability during sample preparation, lack of commercially available analytical standards, and the fact that no published synthetic or analytical methods for the compound exist. This paper reports a method for the preparation of THG via the hydrogenation of gestrinone, and preliminary results on a method for its analysis in equine samples.

Introduction

Tetrahydrogestrinone (THG) described by the FDA as a new and 'purely synthetic designer steroid' derived from gestrinone (Knight 2003), has reportedly been undetected by regulatory laboratories because of decomposition during analysis. Additionally, no certified analytical THG standard has been available, and its synthesis is unreported in the scientific literature. We now report a simple preparation of analytical-standard-quality THG and an analytical method for this substance in biological fluids such as urine or serum.

Materials and methods

Preparation of tetrahydrogestrinone

Preparation of tetrahydrogestrinone (THG, 13-ethyl-17β-hydroxy-18,19-dinor-17α-pregna-4,9,11-trien-3-one, $C_{21}H_{28}O_2$, 312.45 mw) is shown schematically in Figure 1. Tetrahydrogestrinone was prepared by catalytic hydrogenation of the gestrinone ethinyl group. Hydrogenation of gestrinone must be precisely controlled to prevent unwanted hydrogenation at the other unsaturated carbon bonds of gestrinone and various palladium catalysts and hydrogenation reaction conditions were investigated. Stronger catalysts/hydrogenation conditions reduced

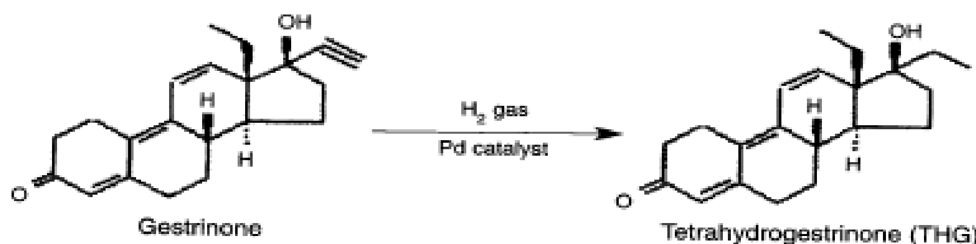

Fig 1: Hydrogenation of gestrinone.

TABLE 1: Gestrinone retention times by GC/MS with temperature programming. Initial temperature held 2 min, then programmed to 280ºC at 20ºC/min

Initial temperature (°C)	Retention time (min)
70	14.8
90	13.8
110	12.8
150	10.8
180	9.3

multiple carbon-carbon double bonds in gestrinone, yielding complex mixtures of reaction products, including norbolethone, a related anabolic steroid (Catlin *et al.* 2002). The following conditions provided satisfactory results.

Gestrinone (100 mg, 0.32 mMol, Sequoia Research Products Ltd) dissolved in 15 ml of toluene was treated with 3 mg of Lindlar catalyst (palladium, 5% on calcium carbonate poisoned with lead, Aldrich). Following air removal, the flask was filled with hydrogen. The reaction mixture was stirred for 36 h under hydrogen at atmospheric pressure, then filtered through celite, after which the toluene was evaporated under reduced pressure. Identification and monitoring of reaction products was by GC/MS of underivatised reaction mixture. At various times during hydrogenation, small aliquots (~1 mg) of reaction mixtures were placed in labelled vials, dried and resuspended in 1 ml ethyl acetate for analysis. Column chromatographic purification on silica gel, with elution by hexane–tert-butyl methyl ether (2:1), yielded 82 mg of THG as a yellowish oil. The yellow oil was subjected to GC/MS analysis on a 30 m x 0.25 mm id x 0.25 μ DB-5MS column (Agilent Corp, Georgia, USA) in an Agilent 6890/5972 GC/MSD. It was decided to temperature programme the column from 180ºC to 280°C at 20°C/min after studying gestrinone behaviour using a range of GC column conditions (Table 1). THG was also subjected to ^{1}H-nmr analysis [^{1}H-nmr (200 MHz, $CDCl_3$) data as follows: δ (ppm) 0.97 (t, 3 H, $\underline{CH_3}CH_2$, J 7.33 Hz), 1.03 (t, 3 H, $\underline{CH_3}CH_2$, J 7.7 Hz), 1.1-2.2 (m, 12 H), 2.4-2.7 (m, 5 H), 2.7-2.9 (m, 2 H), 5.78 (s, 1 H, H-4), 6.37 (d, 1 H, J 10.25 Hz), 6.57 (d, 1 H, J 9.89 Hz)].

Analytical identification

An LC/MS/MS method has been developed for detection, quantitation and confirmation of THG in equine plasma. THG spiked into equine plasma was extracted using liquid-liquid extraction with methyl tert-butyl ether. The dried extract was reconstituted in LC mobile phase and analysed for THG by multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer with an ESI source operated in the positive mode. Specifically, a TSQ Quantum MS (Thermo-Finnigan, California, USA) was equipped with a Surveyor HPLC with an Ace C_8 column (2.1 mm x 50 mm, 5 μm) and guard column (2.1 mm x 12.5 mm, both from Mac-Mod Analytical, Pennsylvania, USA) kept at ambient temperature. Gradient HPLC involved a 10.5 min run, initially at 40% formate buffer (2 mM ammonium formate, pH 3.4): 60% methanol, 300 μl/min, linearly varying to 90% methanol between 4.0 and 8.0 min, returning to 60% methanol at 8.1 min, and increasing flow to 400 μl/min during the last 2.3 min. For quantification, the MS was set to MRM data acquisition, for which parameters were: spray voltage, 4700 V; sheath gas pressure, 43 psi; capillary temperature, 300°C; source CID voltage, -20 V; collision energy, 15 V; scan width, m/z 0.70; scan time 0.4 sec; peak width (FWHM) 0.7 for both quadrupoles Q1 and Q3; and collision gas pressure 1.5 mTorr. Data acquisition and analysis were performed with Xcaliber software v.1.3 (Thermo-Finnigan). The ion transitions monitored for quantitation were m/z 303→187 for the internal standard methenolone, and 313→266 for THG; qualifier ion transitions included m/z 303→187 for methenolone, and 313→225, 313→239 and 313→266 for THG. THG was detectable and quantifiable at concentrations as low as 25 pg/ml and confirmable at 50 pg/ml.

Results

Characterisation of THG by GC/MS analysis

Anecdotal reports have indicated difficulties with the GC analysis of THG. Therefore the satisfactory behaviour of gestrinone during gas chromatography was verified on an Agilent 6890/5972 GC/MSD (Georgia, USA), with separation on a 30 m x 0.25 mm id x 0.25 μ film thickness DB-5MS (5% phenyl-95% methylpolysiloxane) column (J&W Scientific, California, USA). Temperature programmed analyses with step-wise increases in initial column temperatures in the range of 70–180°C yielded single-peak chromatograms for gestrinone (Table 1). Avoidance of derivatisation eliminated the creation of side-products, rendering interpretation of the mass spectra of the reaction mixture compounds less complicated. A rapid assay was required for monitoring the reaction products causing us to opt for the 180°C initial temperature programme. The GC/MS chromatograms showed a number of peaks corresponding to various degrees of hydrogenation (Table 2). THG eluted with adequate separation from other gestrinone

TABLE 2: GC/MS separation of gestrinone hydrogenation products

Molecular weight	Retention time (min)	Identity
308	9.30	Gestrinone [G]
310	8.88	G + 2H
312	9.95	THG
312	10.01	G + 4H
314	10.10	G + 6H
316	9.43	G + 8H

TABLE 3: Principal gestrinone (RT 9.30) and corresponding THG (RT 9.95) EI mass spectral peaks. Below m/z 240 the spectra were virtually indistinguishable.

Gestrinone EI-MS peak (m/z)	Corresponding THG peak (m/z)
308	312
290	294
279	283
261	265
240	240
227	227
211	211

hydrogenation products between 9–10 min retention time under these conditions.

Extracted ion chromatographic analysis (m/z 308, 310, 312, 314, 316 and 318) of the final THG product revealed no 308 mw starting material, no 318 mw fully hydrogenated gestrinone, nor any 312 mw isomeric products. Chromatogram scale expansion revealed minute amounts of 310 (0.7%), 314 (1.0%) and 316 mw (0.16%), verifying that THG was at least 98% pure based on molecular ion areas.

The final THG EI-mass spectrum (Fig 2) revealed only 4 principal high molecular weight ions that differed from corresponding peaks in the gestrinone spectrum (Table 3). These are m/z 312, 294, 283 and 265. This is appropriate because the corresponding peaks in gestrinone (308, 290, 279 and 261, respectively) differed by exactly 4 amu each and have been assigned structures that retain the acetylenic side-group in each case (see fragmentation scheme in Fig 3).

Characterisation of THG by ^{1}H-nmr

Although mass spectral interpretation strongly indicated THG as the principal reaction product, the identification was considered tentative until the addition of the 4 hydrogen atoms was confirmed by proton nuclear magnetic resonance spectroscopy (^{1}H-nmr) as occurring on the ethynyl group attached to C-17. The ^{1}H-nmr spectrum shows 2 different ethyl group signals. Additionally, signals for protons H-4 (singlet), H-11 (doublet) and H-12 (doublet) are identical with the corresponding signals registered for gestrinone, a strong indication that the 3 conjugated carbon-carbon double bonds present in gestrinone remained intact.

Detectability of THG by LC-ESI(+)MS/MS and ELISA

Preliminary efforts in development of an analytical method for analysis of THG in horse

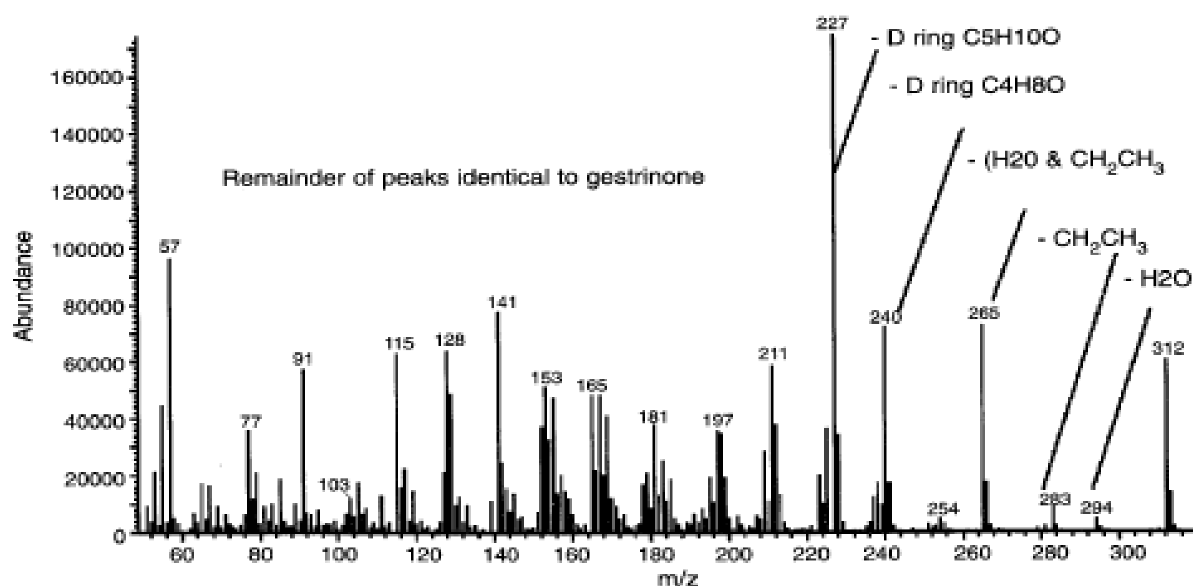

Fig 2: Tetrahydrogestrinone mass spectrum (electron impact).

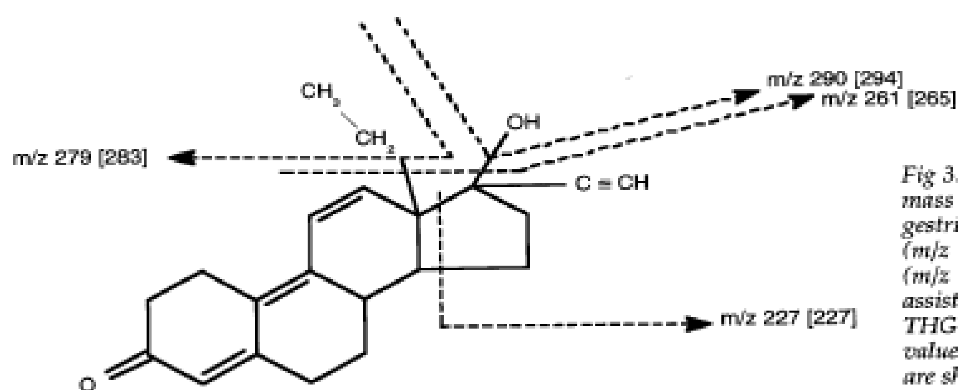

Fig 3: Origin of electron-impact mass spectral fragments in gestrinone that either include (m/z 261, 279, 290) or exclude (m/z 227) the acetylenic group, assisting in the assignment of THG structure. Corresponding values from the THG spectrum are shown in brackets.

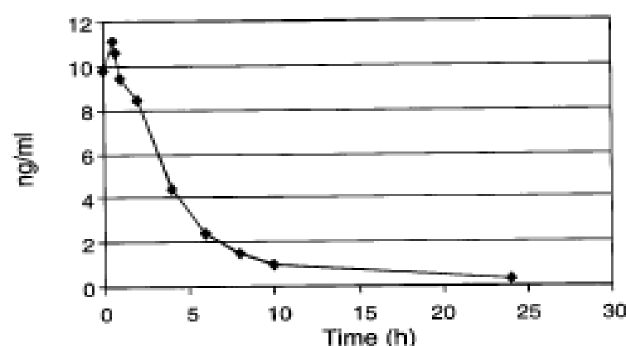

Fig 4: Plasma THG concentrations following im administration of 15 mg THG to a single horse. Concentration at 24 h was about 0.04 ng/ml.

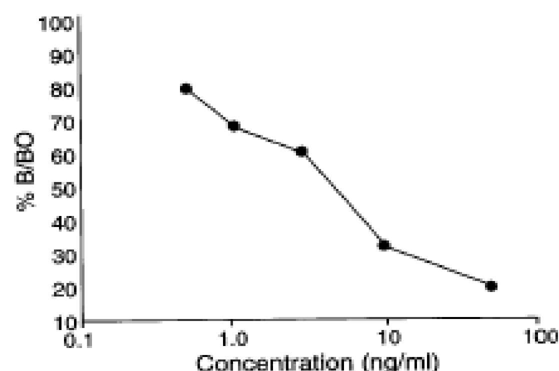

Fig 5: Inhibition curve for THG in buffer using a stanazolol ELISA kit (Neogen Corp). The I_{50} for THG is approximately equivalent to that of stanazolol (not shown).

plasma involved the use of LC-ESI(+)MS/MS. Plasma concentrations shown in Figure 4 indicate a peak value for THG at 30 min following im administration of 15 mg THG in a single horse. Additionally, a Stanazolol ELISA kit (Neogen Corp, Kentucky, USA), tested for cross-reactivity with THG, revealed good detectability with an assay I_{50} of approximately 5 ng/ml (Fig 5).

Discussion

International concern regarding THG has arisen following recent news reports of the use of this 'designer drug' by prominent athletes. Interest in the drug has intensified in the athletic and drug-testing communities, as well as the general public, following reports of prosecution of persons allegedly responsible for its manufacture and distribution.

THG is not commercially available from legitimate sources. In order to obtain an analytical standard for use in the detection and regulation of this drug in horse racing, we have investigated the synthesis of this compound. The most direct preparation of THG is via the hydrogenation of gestrinone. To avoid the formation of a mixture of products and to direct the reaction towards the reduction of only the terminal acetylenic group of gestrinone, different palladium catalysts were tested under varying conditions.

Hydrogenation of gestrinone produced a number of products including, dihydrogestrinone, hexahydrogestrinone, and a compound tentatively identified as norbolethone. Carefully controlled hydrogenation of gestrinone using Lindlar catalyst (Aldrich) produced THG in high yield. The product was purified by column chromatography to give a final product of 98.1% purity.

Should reports of THG use in equine events be forthcoming, we have presented a strategy for its detection and confirmation. ELISA detection with a stanazolol kit from Neogen offers a reasonable detectability, although this needs to be confirmed with samples from dosed horses. LC-ESI(+) MS/MS detection by an MRM protocol following liquid-liquid extraction offered excellent sensitivity and enabled detection of THG up to 24 h post dose.

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Reference

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