The metabolism of anabolic-androgenic steroids in the greyhound

Andrew R. McKinney,[1]* Adam T. Cawley,[2] E. Bruce Young,[3] Carmel M. Kerwick,[3] Karen Cunnington,[3] Rhiannon T. Stewart,[4] Joseph I. Ambrus,[4] Anthony C. Willis[4] and Malcolm D. McLeod[4]

[1] Australian Racing Forensic Laboratory. Currently with the Department of the President's Affairs, PO Box 17292, Al Ain, United Arab Emirates.

[2] Australian Racing Forensic Laboratory, PO Box 528, Kensington, NSW 1465, Australia.

[3] Queensland Government Racing Science Centre, PO Box 513, Albion, QLD 4010, Australia.

[4] Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia.

* Author for correspondence. Tel: +971 3 7686278. Fax: +971 3 7686464. Email: amckinney@mscpc.ae.

Abstract

Background: Effective control of the use of anabolic-androgenic steroids (AASs) in animal sports is essential in order to ensure both animal welfare and integrity. In order to better police their use in Australian and New Zealand greyhound racing, thorough metabolic studies have been carried out on a range of registered human and veterinary AASs available in the region.

Results: Canine metabolic data are presented for the AASs boldenone, danazol, ethylestrenol, mesterolone, methandriol, nandrolone and norethandrolone. The principal phase I metabolic processes observed were the reduction of A-ring unsaturations and/or 3-ketones with either 3α,5β- or 3β,5α-stereochemistry, the oxidation of secondary 17β-hydroxyl groups and 16α-hydroxylation. The phase II β-glucuronylation of sterol metabolites was extensive.

Conclusion: The presented data have enabled the effective analysis of AASs and their metabolites in competition greyhound urine samples.

Key Terms

1. Greyhound racing. A major industry in Australia and New Zealand, greyhound racing in these countries boasts over 30,000 registered participants and attracts over AUD2 billion in wagering annually.

2. Anabolic-androgenic steroids. Despite their infamous reputation as athletic performance enhancers, these substances are also considered to have legitimate therapeutic applications and many racing jurisdictions have adopted policies of control rather than outright bans on their use.

3. Estrus control. Pharmaceutical suppression of the estrus cycle of the racing greyhound bitch is commonplace for husbandry reasons and to maximize the animal's racing career. Low dose AAS administration is the generally preferred mechanism.

4. Racing laboratory. A facility specializing in the chemical analysis of biological samples taken from racing animals for the purpose of doping control. Modern racing laboratories have a heavy reliance on mass spectrometry, commonly supplemented with immunoassay techniques for selected targets.

1 Introduction

The therapeutic use of anabolic-androgenic steroids (AASs) in racing animals is a well established practice. In horse racing, AASs are widely used for the maintenance of condition and appetite and to aid recovery from injury or post-surgical convalescence. In greyhound racing, AASs are the preferred agents for estrus control in bitches, providing strong estrus suppression with few negative side effects when compared to progestins or other available estrus suppressants. In addition to these relatively benign applications, AASs have the potential to alter the physique or psychological nature of a racing animal resulting in enhanced race performance. For this reason, their use in most racing jurisdictions is subject to some form of regulation.

In formulating rules and policies relating to AASs the racing regulator must seek a balance between animal welfare, racing integrity and public perception. The approach adopted by the greyhound racing authorities in Australia and New Zealand has been a total ban on the presence of AASs or their metabolites in animals presented to race with the exception of ethylestrenol, which is permitted in bitches when administered under veterinary supervision for the sole purpose of estrus control. This exemption recognizes the significant complications that may arise from the use of alternative estrus suppressants and the undesirability of standing a bitch down from racing whilst in heat. This policy was implemented across Australia between July and October 2008 and in New Zealand in January 2010.

The new regulations posed a significant challenge for the Australasian racing laboratories. AASs typically undergo extensive phase I and II metabolism *in vivo* and a thorough knowledge of the relevant metabolic pathways is essential for effective doping control. However, despite a wealth of publications describing human [1,2 and references cited therein] and equine [3 and references cited therein] AAS metabolism, there is very little published canine data [4-9]. To help rectify this situation, the industry initiated a program of scientific research aimed at providing the information and resources required for effective testing. The program involved a series of controlled canine AAS administration trials followed by extensive chemical analysis to elucidate the major metabolic pathways and identify suitable analytical targets for doping control. Furthermore, where strategic targets were found to be

commercially unavailable, efforts were made to synthesize authentic samples for use as reference standards. Priority was given to AASs which were locally available either as human or veterinary preparations. Thus, investigations have been carried out for boldenone undecylenate, danazol, ethylestrenol, mesterolone, methandriol dipropionate, nandrolone laurate, norethandrolone and stanozolol. During each investigation, useful forensic markers were unambiguously identified and, where otherwise unavailable, synthesized and characterized. Stanozolol is the subject of a separate publication [10]. The remaining data is presented herein.

2 Experimental

2.1 Purchased Steroids

19-Norepiandrosterone (3β-hydroxy-5α-estran-17-one) was purchased from Alltech (NSW, Australia). 17-Epiboldenone (17α-hydroxyandrosta-1,4-dien-3-one), 17-epinandrolone (17α-hydroxyestr-4-en-3-one), ethylestrenol (19-nor-17α-pregn-4-en-17β-ol), 17β-hydroxy-5β-androst-1-en-3-one, 3α-hydroxy-1α-methyl-5α-androstan-17-one, 1α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5β-androstane-3α,17β-diol and 19-noretiocholanolone (3α-hydroxy-5β-estran-17-one) were purchased from the National Measurement Institute (NSW, Australia). Androsta-1,4-diene-3,17-dione, androsterone (3α-hydroxy-5α-androstan-17-one), boldenone (17β-hydroxyandrosta-1,4-dien-3-one), danazol (isoxazolo[4',3':2,3]-17α-pregn-4-en-20-yn-17β-ol), epiandrosterone (3β-hydroxy-5α-androstan-17-one), estr-4-ene-3,17-dione, ethisterone (17β-hydroxy-17α-pregn-4-en-20-yn-3-one), etiocholanolone (3α-hydroxy-5β-androstan-17-one), methandriol (17α-methylandrost-5-ene-3β,17β-diol), 17α-methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one), nandrolone (17β-hydroxyestr-4-en-3-one) and norethandrolone (17β-hydroxy-19-nor-17α-pregn-4-en-3-one) were purchased from Sigma (NSW, Australia). 5α-Androst-1-ene-3,17-dione, 3-epietiocholanolone (3β-hydroxy-5β-androstan-17-one), 5α-estrane-3α,17α-diol, 5α-estrane-3α,17β-diol, 5α-estrane-3β,17α-diol, 5α-estrane-3β,17β-diol, 5β-estrane-3α,17α-diol, 5β-estrane-3α,17β-diol, 5β-estrane-3β,17β-diol, 17β-hydroxy-5α-androst-1-en-3-one, mesterolone (17β-hydroxy-1α-methyl-5α-androstan-3-one), 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5α-androstane-3β,17β-diol were purchased from Steraloids (RI, USA).

2.2 Synthesized Steroids

2.2.1 5β-Androst-1-ene-3,17-dione

The title compound was formed by oxidation of the corresponding 17β-alcohol.

Pyridinium chlorochromate (5 mg) was added to a solution of 17β-hydroxy-5β-androst-1-en-3-one (1 mg) in dichloromethane (4 mL) and mixed by rotation for 4 h at room temperature. Excess oxidizing agent was destroyed by the addition of sodium bisulfite solution (0.1 M, 4 mL) and the organic phase was dried over anhydrous sodium sulfate and dried by evaporation at 60 °C under nitrogen.

2.2.2 5α-Androst-1-ene-3α,17β-diol and 5α-androst-1-ene-3β,17β-diol

The title 3-epimeric diols were prepared by a simple reduction of the corresponding 3-ketone. The lithium aluminum hydride reduction of the Δ^1-5α-androstan-3-one A-ring has been described by Bergmann *et al.* [11] and strongly favours the formation of the 3β-alcohol.

Lithium aluminum hydride (5 mg) was added to a solution of 17β-hydroxy-5α-androst-1-en-3-one (1 mg) in diethyl ether (4 mL) and mixed by rotation for 1 h at room temperature. Excess reducing agent was destroyed by the dropwise addition of 2-propanol followed by sulfuric acid (1 M, 4 mL) and the organic phase was dried by evaporation at 60 °C under nitrogen. GC-MS analysis revealed the existence of two peaks in 10.1:1 ratio which were assigned as the 3β- and 3α-alcohols respectively in accordance with literature precedent [11].

2.2.3 5β-Androst-1-ene-3α,17β-diol and 5β-androst-1-ene-3β,17β-diol

The title 3-epimeric diols were prepared from 17β-hydroxy-5β-androst-1-en-3-one in an analogous manner to the 5α-isomers described above (section 2.2.2). In this case it is the 3α-product which predominates [12], and two GC-MS peaks in 6.5:1 ratio

were assigned as the 3α- and 3β-alcohols respectively in accordance with the literature precedent.

2.2.4 5β-Estrane-3β,17α-diol

5β-Estrane-3β,17α-diol was prepared from its 3α,17β-isomer by a Mitsunobu reaction with simultaneous stereochemical inversion at both C3 and C17.

Diisopropyl azodicarboxylate (1.3 μL) was added to a suspension of 5β-estrane-3α,17β-diol (1 mg) in a solution of 4-nitrobenzoic acid (1.1 mg) and triphenylphosphine (1.7 mg) in anhydrous ether (0.5 mL). The mixture was shaken until the steroid had completely dissolved (approx. 5 min), then was allowed to stand overnight at room temperature. The solution was diluted with ethyl acetate (5 mL), washed with sodium carbonate solution (1 M, 5 mL) and the organic fraction was dried by evaporation at 60 °C under a stream of nitrogen. The 4-nitrobenzoylated residue was reconstituted in methanol (1 mL) and sodium hydroxide solution (10 M; 1 drop) was added. The solution was allowed to stand overnight at room temperature, then was diluted with hydrochloric acid (1 M, 5 mL) and extracted with ethyl acetate (5 mL). The organic fraction was dried by evaporation at 60 °C under a stream of nitrogen. The residue was purified by chromatography on a short silica column using ethyl acetate:dichloromethane (1:1 v/v) and the product was shown to be chromatographically resolved by GC from its three commercially available C3/C17 stereoisomers.

2.2.5 1α-Methyl-5α-androstane-3β,17β-diol

Mesterolone contains a 1α-methyl substituent oriented axially on the 5α-androstane steroid framework. As previously described [13], this group blocks the lower face of the 3-ketone such that hydride reduction affords mixtures of the 3α- and 3β-alcohols. Sodium borohydride reduction of the mesterolone gave a 1:1 mixture of the two alcohols on a small scale. Greater selectivity for the 3β-alcohol was afforded in this case by reduction with sodium in ethanol, giving the desired isomer in 70% yield.

To a solution of mesterolone (30 mg) in ethanol (10 mL) was carefully added excess sodium metal. The solution was heated to reflux and the reaction was monitored by thin layer chromatography. On completion, the solution was cooled and ethanol (10 mL) was slowly added followed by water (30 mL). The ethanol was evaporated under reduced pressure and the residual mixture was extracted with ethyl acetate (3 x 10 mL). The extract was dried by evaporation under reduced pressure and the crude material was purified by chromatography on silica to give the pure product (21 mg, 70%) as a white solid: R_f 0.49 (40% ethyl acetate in hexane); $[\alpha]^{20}_D$ +17 (*c* 1.40, $CHCl_3$) (lit. [13] $[\alpha]^{29}_D$ +18 (*c* 1.0, $CHCl_3$)); IR (thin film) 3420 (O-H), 2957, 2924, 2851 (C-H) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.76 (1H, tt, *J* 11.7, 4.8 Hz, *H*3), 3.56 (1H, t, *J* 8.4 Hz, *H*17), 2.08-1.89 (1H, m), 1.86-1.73 (2H, m), 1.71-1.37 (10H, m), 1.35-1.24 (6H, m), 1.03-0.85 (4H, m), 0.95 (3H, s, CH_3), 0.94 (3H, d, *J* 7.8Hz, CH_3), 0.74 (3H, s, CH_3); ^{13}C NMR (75.45 MHz, $CDCl_3$) δ 82.0, 67.0, 51.2, 48.9, 43.2, 38.9, 38.6, 37.8, 37.6, 37.2, 36.8, 35.7, 31.5, 30.5, 28.8, 23.5, 20.2, 15.1, 14.4, 11.3; *m/z* (+EI) 306 ($M^{\bullet+}$, 5%), 288 (4), 234 (6), 220 (6), 206 (6), 191 (41), 149 (15), 97 (20), 83 (76), 49.0 (100); HRMS (+EI) calcd for $C_{20}H_{34}O_2$ ($M^{\bullet+}$) 306.2559 found 306.2560.

2.2.6 17α-Methyl-5β-androstane-3α,16α,17β-triol

The title compound was synthesized from etiocholanolone by a modification of the method of Watabe *et al.* [14] and Leslie [15].

2.2.6.1 3α,17-Diacetoxy-5β-androst-16-ene [15]

To a solution of etiocholanolone (1.5 g, 5.16 mmol) in isopropenyl acetate (25 mL) was added concentrated sulfuric acid (2 drops). The solution was heated in a distillation apparatus until the reaction volume had reduced by approximately half. The isopropenyl acetate was restored to its original volume and heating was continued. This concentration-dilution sequence was repeated for 5 h. The reaction mixture was diluted with diethyl ether (25 mL) and the organic layer was washed with saturated aqueous sodium hydrogen carbonate solution (3 x 10 mL) and brine (25 mL). The organic layer was dried ($MgSO_4$) and concentrated to give a brown solid. The crude material was purified by flash column chromatography using 10% ethyl

acetate in hexane to give the title compound (1.28 g, 66%) as a white solid: R_f = 0.60 (25% ethyl acetate in hexane); m.p. 89-90 °C (lit. [15] 90-91 °C); $[\alpha]^{20}_D$ +62.1 (*c* 1.0, CH_3OH); IR (thin film) 2932, 2866 (C-H), 1734 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.44 (1H, m, *H*16), 4.71 (1H, m, *H*3), 2.15 (1H, m), 2.14 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$), 1.93-0.98 (19H, m), 0.95 (3H, s, CH_3) 0.86 (3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.9, 169.2, 159.9, 111.6, 74.4, 54.4, 45.1, 42.2, 41.3, 35.2, 30.1, 34.2, 33.8, 32.5, 29.1, 27.1, 26.8, 25.8, 23.5, 21.7, 21.4, 20.5, 15.8; HRMS (+EI) calcd for $C_{23}H_{34}O_4$ ($M^{\bullet+}$) 374.2457, found 374.2450.

2.2.6.2 3α,16α-Diacetoxy-5β-androstan-17-one [14]

To a solution of 3α,17-diacetoxy-5β-androst-16-ene (381.3 mg, 1.02 mmol) in dichloromethane (10 mL) was added *meta*-chloroperoxybenzoic acid (527.1 mg, 3.05 mmol) in one portion. The reaction was stirred at room temperature for 1 h, at which point consumption of the starting material was indicated by TLC. The reaction mixture was washed with aqueous sodium hydroxide (1 M , 3 x 10 mL) and the organic layer was dried ($MgSO_4$) and concentrated to give a white foam, which was used without further purification in the next step. The crude epoxide was dissolved in methanol (5 mL) and aqueous sulfuric acid (5 mL, 3 M) and stirred for 1 h at room temperature, after which TLC analysis showed consumption of the starting material. The reaction was diluted with ethyl acetate (20 mL) and extracted with aqueous sodium hydroxide (1 M, 3 x 10 mL). The organic layer was dried ($MgSO_4$) and concentrated to give a white solid, which was used without further purification in the next step. The crude ketone was dissolved in acetic anhydride (5 mL) and pyridine (1 mL) and the reaction was heated to 80 °C for 1.5 h, at which point consumption of the starting material was indicated by TLC. The crude reaction mixture was diluted with diethyl ether (20 mL) and washed with aqueous sodium hydroxide (1 M, 3 x 10 mL), aqueous hydrochloric acid (3 x 10 mL, 1 M) and brine (10 mL). The organic layer was dried ($MgSO_4$) and concentrated to give a white solid which was purified by flash column chromatography using 10% ethyl acetate in hexane to give the title compound (317.8 mg, 80% over 3 steps) as a white solid: $[\alpha]^{20}_D$ +75.3 (*c* 1.0, $CHCl_3$); IR (thin film) 2937, 2866 (C-H), 1757, 1738 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.36 (1H, dd, *J* 9.2, 0.8 Hz, *H*16), 4.69 (1H, m, *H*3), 2.15-1.00 (20H, m,), 2.08 (3H, s,

$COCH_3$), 1.99 (3H, s, $COCH_3$), 0.931 (3H, s, CH_3), 0.926 (3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 214.1, 170.7, 170.4, 74.0, 72.6, 48.8, 47.8, 41.8, 40.7, 35.3, 35.0, 34.8, 32.2, 31.6, 29.7, 26.7, 26.6, 25.0, 23.3, 21.5, 20.9, 19.8, 14.3; HRMS (+EI) calcd for $C_{23}H_{34}O_5$ ($M^{\bullet+}$) 390.2406, found 390.2408.

2.2.6.3 17α-Methyl-5β-androstane-3α,16α,17β-triol [14]

A solution of 3α,16α-diacetoxy-5β-androstan-17-one (93.2 mg, 0.24 mmol) in diethyl ether (5 mL) was cooled to -78 °C and a solution of methylmagnesium bromide in ether (3 M, 1.99 mL, 5.97 mmol) was added. The reaction was maintained at -78 °C for 5 min before warming to 40 °C for 2 h. The crude reaction mixture was poured onto saturated ammonium chloride solution (25 mL) and diluted with ethyl acetate (20 mL). The organic layer was separated, dried ($MgSO_4$) and concentrated to give a white solid. The crude product was purified by flash column chromatography using a gradient elution of 20%-40%-60%-80% ethyl acetate in hexane to afford (1) 17β-methyl-5β-androstane-3α,16α,17α-triol as a white solid (13.6 mg, 18%): R_f 0.25 (ethyl acetate); m.p. 238-240 °C (lit. [14] 240-241 °C); $[\alpha]^{20}_D$ -25.3 (*c* 1.0, CH_3OH); IR (thin film) 3351 (O-H), 2982, 2862 (C-H), 1735 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 4.01 (1H, dd, *J* 2.8, 9.2 Hz, *H*16), 3.53 (1H, dddd, *J* 4.8, 4.8, 10.8, 10.8 Hz, *H*3), 1.95-1.62 (7H, m), 1.53-1.15 (12H, m), 1.12 (3H, s, CH_3), 1.01 (1H, m), 0.95 (3H, s, CH_3), 0.70 (3H, s, CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 81.2, 87.1, 72.4, 49.4, 48.0, 43.6, 41.9, 37.2 (2C), 36.5, 35.8, 35.6, 31.7, 31.2, 28.2, 27.9, 24.0, 20.9, 20.3, 16.3; HRMS (EI+) calcd for $C_{20}H_{34}O_3$ 322.2508, found 322.2502; and (2) 17α-methyl-5β-androstane-3α,16α,17β-triol as a white solid (31.1 mg, 40%): R_f 0.11 (ethyl acetate); m.p. 216-218 °C (lit. [14] 220-221 °C); $[\alpha]^{20}_D$ -2.6 (*c* 1.32, CH_3OH); IR (thin film) 3339 (O-H), 2921, 2857 (C-H) cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 4.17 (1H, dd, *J* 3.2, 10.8 Hz, *H*16), 3.54 (1H, m, *H*3), 1.95-1.72 (4H, m), 1.64 (1H, m), 1.51-1.26 (14H, m), 1.15 (3H, s, CH_3), 1.01 (1H, m), 0.96 (3H, s, CH_3), 0.84 (3H, s, CH_3); ^{13}C NMR (100 MHz, CD^3OD) δ 85.0, 80.7, 72.3, 49.6, 47.5, 43.6, 42.0, 37.7, 37.2, 36.4, 35.8, 34.8, 33.3, 31.1, 28.3, 37.4, 23.9, 21.1, 18.2, 15.0; HRMS (+EI) calcd for $C_{20}H_{34}O_3$ 322.2508, found 322.2508. The stereochemistry of the minor product (1) was confirmed by single crystal X-ray analysis [16].

2.2.7 19-Nor-5β,17α-pregnane-3α,16α,17β-triol and 19-nor-5β,17β-pregnane-3α,16α,17α-triol

The title compound was synthesized on a small scale from 19-noretiocholanolone by a route analogous to that described above (section 2.2.6), with the exception that the 17β-ethyl substituent was introduced *via* the 17-ketone through a process of ethynylation with lithium acetylide ethylenediamine complex followed by hydrogenation [17]. GC-MS analysis revealed the existence of two peaks in 10:1 ratio which were assigned as the 3α,16α,17β- and 3α,16α,17α-triols respectively based on the susceptibility of the latter to 16,17-isopropylidene acetal formation [18].

Syntheses of all four 19-nor-5,17α-pregnane-3,17β-diol C3/C5 isomers, both 19-nor-5α,17α-pregnane-3β,16,17β-triol C16 isomers and both 19-nor-5α,17α-pregnane-3β,17β,20-triol C20 isomers have been reported previously [19], as have syntheses of both 17α-methyl-5β-androstane-3,17β-diol C3 isomers and all eight 17α-methylandrostane-3,16,17β-triol C3/C5/C16 isomers [18].

2.3 Animal Administrations

All animal administration experiments were approved by the Queensland Department of Primary Industries and Fisheries Community Access Animal Ethics Committee. Urine samples were collected by spontaneous voiding and were immediately frozen and stored at -20 °C until required for analysis.

2.3.1 Boldenone

An oil solution of boldenone undecylenate (Boldebal-H®, Ilium, NSW, Australia; 1.1 mL = 55 mg boldenone undecylenate) was administered by intramuscular injection to a female greyhound (9 years, 27 kg). Urine samples were collected pre-administration and from 6 h-29 days post-administration.

2.3.2 Danazol

Danazol (Azol 100®, Alphapharm, NSW, Australia; 1 tablet = 100 mg danazol) was administered orally to a female greyhound (3.5 years, 23 kg). Urine samples were collected pre-administration and from 2 h-4 days post-administration.

2.3.3 Ethylestrenol

Ethylestrenol (Nandoral®, Intervet, VIC, Australia; 2 tablets = 1 mg ethylestrenol) was administered orally to a female greyhound (2.5 years, 26 kg). Urine samples were collected pre-administration and from 2 h-7 days post-administration.

2.3.4 Mesterolone

Mesterolone (Proviron®, Bayer Schering, NSW, Australia; 1 tablet = 25 mg mesterolone) was administered orally to a female greyhound (1.5 years, 29 kg). Urine samples were collected pre-administration and from 2 h-4 days post-administration.

2.3.5 Methandriol

An oil solution of methandriol dipropionate (Anadiol Depot®, Ilium Veterinary Products, NSW, Australia; 1 mL = 75 mg methandriol dipropionate) was administered by intramuscular injection to a male greyhound (2 years, 35 kg). Urine samples were collected pre-administration and from 7 h-105 days post-administration.

2.3.6 Nandrolone

An oil solution of nandrolone laurate (Laurabolin®, Intervet/Schering-Plough Animal Health, VIC, Australia; 2 mL = 50 mg nandrolone laurate) was administered by intramuscular injection to a female greyhound (3 years, 27 kg). Urine samples were collected pre-administration and from 7 h-146 days post-administration.

2.3.7 Norethandrolone

Norethandrolone (Anaplex®, Jurox, NSW, Australia; 2.5 tablets = 12.5 mg norethandrolone) was administered orally to a female greyhound (4 years, 23 kg). Urine samples were collected prior to administration, then at 2, 4, 6, 8, 24, 32, 48, 56 and 72 h post-administration.

2.4 Sample Preparation2.4.1 Solid Phase Extraction

Aliquots of urine (3 mL) were adjusted to pH 7 and centrifuged to sediment particulate matter. The supernatant fractions were loaded onto Waters Oasis WAX solid phase extraction cartridges (3 mL, 60 mg, 60 µL; P/N 186002492) which had previously been conditioned with methanol (1 mL) and water (3 mL). The cartridges were washed with sodium hydroxide solution (0.1 M, 3 mL), sodium phosphate buffer solution (0.05 M, pH 7.5, 3 mL) and water (3 mL), then were dried under vacuum to remove residual water. The cartridges were sequentially eluted with methanol:ethyl acetate (1:1 v/v, 3 mL; unconjugated fraction), methanol:ethyl acetate:formic acid (50:50:1 v/v/v, 3 mL; β-glucuronide conjugated fraction) and methanol:ethyl acetate:diethylamine (50:50:1 v/v/v, 3 mL; sulfate conjugated fraction) with several minutes drying under vacuum between the second and third elutions. All three eluates were dried by evaporation at 80 °C under nitrogen.

2.4.2 Cleanup: Unconjugated Fraction

Dried eluates were reconstituted in methanol (0.5 mL) and sodium hydroxide solution (2 M, 4 mL) and extracted with diisopropyl ether (4 mL). The extracts were dried by evaporation at 80 °C under nitrogen.

2.4.3 Hydrolysis and Cleanup: β-Glucuronide Conjugated Fraction

Dried eluates were reconstituted in sodium citrate buffer solution (0.1 M, pH 6, 0.5 mL) and *Escherichia coli* β-glucuronidase solution (Sigma Type IX-A lyophilized powder, P/N G7396; 2 mg/mL, 50 µL) and incubated overnight at 37 °C. Sodium

hydroxide solution (2 M, 4 mL) was added and the mixture was extracted with diisopropyl ether (4 mL). The extracts were dried by evaporation at 80 °C under nitrogen.

2.4.4 Solvolysis and Cleanup: Sulfate Conjugated Fraction

Dried eluates were reconstituted in anhydrous methanolic hydrogen chloride (1 M, 0.5 mL) [20] and incubated for 15 min at 60 °C. Sodium hydroxide solution (2 M, 4 mL) was added and the mixture was extracted with diisopropyl ether (4 mL). The extracts were dried by evaporation at 80 °C under nitrogen.

2.4.5 Derivatisation: Oximination/tert-Butyldimethylsilylation

Dried residues were reconstituted in a solution of hydroxylamine hydrochloride in pyridine (10% w/v, 50 µL) and incubated for 30 min at 100 °C. *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (50 µL) was added and incubated for a further 60 min at 100 °C. The reaction was quenched by the addition of water (2 mL) and the mixture was extracted with hexane (2 mL). The extracts were dried by evaporation at 80 °C under nitrogen.

2.4.6 Derivatisation: O-Methyloximination/tert-Butyldimethylsilylation

Dried residues were reconstituted in a solution of methoxylamine hydrochloride in pyridine (2% w/v, 50 µL) and incubated for 30 min at 100 °C. A solution of *tert*-butyldimethylsilyl trifluoromethanesulfonate in *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (1% v/v; 50 µL) was added and incubated for a further 60 min at 100 °C. The reaction was quenched by the addition of water (2 mL) and the mixture was extracted with hexane (2 mL). The extracts were dried by evaporation at 80 °C under nitrogen.

2.4.7 Derivatisation: O-Methyloximination/Trimethylsilylation

Dried residues were reconstituted in a solution of methoxylamine hydrochloride in pyridine (2% w/v, 50 µL) and incubated for 30 min at 100 °C. The solutions were dried by evaporation at 80 °C, then were reconstituted in a solution of ammonium iodide and 1,4-dithioerythritol in *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (0.2% and 0.4% w/v; 50 µL) and incubated for 60 min at 100 °C. The solutions were dried by evaporation at 80 °C under nitrogen.

2.5 GC-MS Analyses

Derivatized residues were reconstituted in dodecane (50 µL) for GC-MS analysis. Data were acquired using a Waters-Micromass Quattro triple quadrupole mass spectrometer interfaced to an Agilent 6890 gas chromatograph equipped with a SGE BPX-5 column (30 m x 0.25 mm I.D. x 0.25 µm film) and using helium as carrier gas. Sample injections (2 µL) were made in splitless mode with an injector temperature of 200 °C. The column was held at 100 °C for 1 min, then was ramped at 10 °C/min to 300 °C and held for a further 5 min. The column flow was maintained at 1 mL/min throughout the run and the purge valve was opened at 2 min to a split of 30:1. The detector was operated in electron ionization mode with a transfer line temperature of 250 °C, source temperature of 200 °C and ionization energy of 70 eV. Full scan data were collected over the range *m/z* 50-700 with a scan time of 250 ms.

3 Results & Discussion

Post-administration urine samples were extracted as described above and subjected to three different derivatization procedures prior to GC-MS analysis. Putative metabolites were identified on the basis of their mass spectral characteristics and, where possible, confirmed against authentic reference standards. In the absence of any exclusively unconjugated 17α-alkyl metabolites, the formation of 17-epimerized artifacts from 17-sulfated 17α-alkyl-17β-hydroxy metabolites, as originally described by Edlund *et al.* [21], was not considered.*3.1 Boldenone*

The canine metabolism of boldenone has been examined both *in vitro* and *in vivo* by Williams *et al.* [4,5]. Phase I pathways identified after incubation with canine liver

microsomes included oxidation of the 17-hydroxyl group and hydroxylation at one or more other positions. *In vivo*, 41 urinary phase I metabolites arising from various permutations of single bond oxidation, double bond reduction and hydroxylation were observed after oral administration. Following intravenous administration, Brockwell *et al.* [6] reported the presence in urine of 5α-androstane-3β,17α-diol and 5α-androstane-3β,17β-diol.

In the present study, boldenone was administered intramuscularly as its long acting undecylenate ester. Eight metabolites were detected in urine [7]. Analytes confirmed by comparison with authentic reference standards were boldenone, androsta-1,4-diene-3,17-dione, 5β-androst-1-ene-3,17-dione, epiandrosterone, 17β-hydroxy-5β-androst-1-en-3-one and 5β-androst-1-ene-3α,17β-diol. In addition, two compounds showing mass spectra consistent with a dihydroxyandrostenone and an androstenetriol were detected but could not be definitively identified due to the unavailability of reference standards. 17-Epiboldenone, 5α-androst-1-ene-3,17-dione, androsterone, etiocholanolone, 3-epietiocholanolone, 17β-hydroxy-5α-androst-1-en-3-one, 5α-androst-1-ene-3α,17β-diol, 5α-androst-1-ene-3β,17β-diol and 5β-androst-1-ene-3β,17β-diol were not detected. Whole or partial phase II β-glucuronylation was observed for all of the observed hydroxylated metabolites, while non-hydroxylated metabolites were unconjugated. No sulfate conjugated metabolites were detected. The detection time for some metabolites was in excess of 4 weeks following a single dose.

The above pattern of phase I metabolites may be explained by a combination of 17-oxidation, complete reduction of the $\Delta^{1,4}$-3-ketone with 3β,5α-stereochemistry, partial reduction of the $\Delta^{1,4}$-3-ketone with 3α,5β-stereochemistry and hydroxylation at an unidentified site (Fig. 1).

3.2 Danazol

The canine metabolism of danazol has not previously been reported, but ethisterone is a known major metabolite in humans, monkeys, rats [22] and horses [23] and was also detected in greyhound urine after oral danazol administration (Fig. 2). Extraction and derivatisation by *O*-methyloximination and *tert*-butyldimethylsilylation revealed

ethisterone with a detection time of about 1 week. Most of the excreted ethisterone was β-glucuronide conjugated, the remainder being unconjugated.

3.3 Ethylestrenol

The canine metabolism of ethylestrenol has not previously been reported. In humans and monkeys it is metabolized by oxidation to norethandrolone followed by reduction of the Δ^4-3-ketone and hydroxylation at C21 [24]; and in the rat [25] and horse [26,27] by a similar sequence of oxidation and reduction.

The oral administration of ethylestrenol to a female greyhound allowed the detection of several potential urinary metabolites, but most were too weak to allow confident mass spectral interpretation. The strongest peak appeared to correspond to a 19-norpregnanetriol with no additional hydroxylation on the D-ring or ethyl side chain (Fig. 3): the tris(*tert*-butyldimethylsilyl) derivative showed *m/z* 607 ($[M\text{-}t\text{Bu}^{\bullet}]^+$), 532 ($[M\text{-}t\text{BuMe}_2\text{SiOH}]^{\bullet+}$), 477 ($[M\text{-}t\text{Bu}^{\bullet}\text{-}\text{Me}_2\text{SiO-}\text{C}_4\text{H}_8]^+$), 269 ($[M\text{-}t\text{Bu}^{\bullet}\text{-}\text{Me}_2\text{SiO-}2t\text{BuMe}_2\text{SiOH}]^+$), 199 (D-ring) and 186 (D-ring); the tris(trimethylsilyl) derivative showed *m/z* 538 ($M^{\bullet+}$), 509 ($[M\text{-}\text{Et}^{\bullet}]^+$), 448 ($[M\text{-}\text{Me}_3\text{SiOH}]^{\bullet+}$), 419 ($[M\text{-}\text{Et}^{\bullet}\text{-}\text{Me}_3\text{SiOH}]^+$), 358 ($[M\text{-}2\text{Me}_3\text{SiOH}]^{\bullet+}$), 268 ($[M\text{-}3\text{Me}_3\text{SiOH}]^{\bullet+}$), 239 ($[M\text{-}\text{Et}^{\bullet}\text{-}3\text{Me}_3\text{SiOH}]^+$), 157 (D-ring) and 144 (D-ring) (Fig. 4). Clearly this compound differs from the identified 19-norpregnanetriol metabolites of the related steroid norethandrolone (section 3.7). Ethylestrenol and norethandrolone were not detected, nor were any 19-nor-5,17α-pregnane-3,17β-diols (4 isomers), 19-nor-5α,17α-pregnane-3β,16,17β-triols (2 isomers) or 19-nor-5α,17α-pregnane-3β,17β,20-triols (2 isomers). The major metabolite was β-glucuronide conjugated and could be detected for 72 hours post-administration.

3.4 Mesterolone

The canine metabolism of mesterolone has not previously been reported. In humans it is metabolized by reduction at C3 and oxidation at C17 [12] and in the horse by reduction at C3, hydroxylation at C16 and C18 and epimerization at C17 [28].

Following the oral administration of mesterolone to a greyhound, the presence of 7 metabolites was revealed. Most could not be definitively identified due to a lack of reference standards, but the most intense metabolite peak was confirmed as 1α-methyl-5α-androstane-3β,17β-diol (Fig. 5) using a synthesized standard (section 2.2.5). The metabolites of unconfirmed structure gave mass spectra consistent with isomers of mesterolone (1 isomer), monohydroxylated mesterolone (3 isomers) and oxidized monohydroxylated mesterolone (2 isomers). Mesterolone, 1α-methyl-5α-androstane-3α,17β-diol and 3α-hydroxy-1α-methyl-5α-androstan-17-one were not detected. Phase II metabolism took the form of β-glucuronylation with a minor degree of sulfation. No unconjugated metabolites were detected. The detection time for the major metabolite was about 48 hours.

3.5 Methandriol

The canine metabolism of methandriol has not previously been reported. In humans the major metabolite after oral administration is 17α-methyl-5β-androstane-3α,17β-diol [12], while in the horse it is metabolized by reduction of the Δ^5-unsaturation and hydroxylation at C16 and C19 [29,30].

In this work, the long acting methandriol dipropionate was administered by intramuscular injection. 3 urinary phase I metabolites were detected: methandriol, 17α-methyl-5α-androstane-3β,17β-diol and 17α-methyl-5β-androstane-3α,16α,17β-triol [8]. The triol, identified using synthesized reference standards and previously described GC-MS data [18], was the major metabolite and was detectable for 90 days post-administration. It was subsequently synthesized on a larger scale and fully characterized (section 2.2.6). No 17α-methyltestosterone or other isomeric 17α-methyl-5-androstane-3,17β-diols (3 isomers) or 17α-methyl-5-androstane-3,16,17β-triols (7 isomers) were observed. All metabolites were β-glucuronide conjugated. The saturated metabolites are the same as are observed in the greyhound for following 17α-methyltestosterone administration [9] suggesting the latter may be a metabolic intermediate (Fig. 6), although it was not observed in this case.

3.6 Nandrolone

Brockwell *et al.* [6] have previously reported the detection of nandrolone, estr-4-ene-3,17-dione, 19-norepiandrosterone, 19-noretiocholanolone and some uncharacterized reduced and hydroxylated metabolites in urine following the intravenous administration of nandrolone to a greyhound.

In the present study, an oil solution of the long acting nandrolone laurate was administered by intramuscular injection. A total of 6 metabolites was observed: nandrolone, 19-norepiandrosterone, 19-noretiocholanolone, 5α-estrane-3β,17α-diol, 5α-estrane-3β,17β-diol and 5β-estrane-3α,17β-diol [8]. Estr-4-ene-3,17-dione, 17-epinandrolone and 5β-estrane-3β,17β-diol were not detected, nor was there any evidence of the presence of other estrane-3,17α-diols (3 isomers). 5α-Estrane-3β,17β-diol and 5β-estrane-3α,17β-diol were the major metabolites and were detectable for over 140 days. All metabolites were predominantly β-glucuronide conjugated. The above pattern may be explained by a combination of Δ^4-3-ketone reduction with 3α,5β- or 3β,5α-stereochemistry, oxidation of the 17β-hydroxyl and reduction of the resulting 17-ketone with 17α-stereochemistry (Fig. 7).

3.7 Norethandrolone

The canine metabolism of norethandrolone has not previously been reported. In humans it is metabolized primarily by reduction of the Δ^4-3-ketone and hydroxylation at C21 [24]; in marmoset monkeys by reduction and hydroxylation of the B-ring [24]; in cows by reduction [31] and in the horse by reduction, hydroxylation at C16 and C20 and full oxidation at C21 [19,32,33].

Following the oral administration of norethandrolone to a greyhound, five phase I urinary metabolites were detected. Of these two were identified by comparison with synthesized reference standards as 19-nor-5α,17α-pregnane-3β,17β-diol and (20*R*)-19-nor-5α,17α-pregnane-3β,17β,20-triol and a third was tentatively identified as 19-nor-5β,17α-pregnane-3α,16α,17β-triol using a partially characterised synthesised reference material (section 2.2.7). The diol metabolite was detectable for 24 h and the triols for 56 h post-administration. No other isomeric 19-nor-5,17α-pregnane-3,17β-diols (3 isomers) were observed, nor were 19-nor-5α,17α-pregnane-3β,16α,17β-triol, 19-nor-5α,17α-pregnane-3β,16β,17β-triol or (*20S*)-19-nor-5α,17α-

pregnane-3β,17β,20-triol. The remaining uncharacterized metabolites showed mass spectra consistent with a 19-norpregnane-3,17,20-triol and a 19-norpregnanetriol hydroxylated at C3, C17 and an additional site not on the D-ring or ethyl side chain. The 19-nor-5β,17α-pregnane-3α,16α,17β-triol and 19-norpregnane-3,17,20-triol metabolites displayed a mixture of β-glucuronide and sulfate conjugation, while the remaining metabolites were all present as β-glucuronides exclusively. This metabolism (Fig. 8) is analogous in some respects to that observed for methandriol and 17α-methyltestosterone (Fig. 7) but, interestingly, seems different to that observed for ethylestrenol (section 3.3), which acts as a norethandrolone prodrug in other species.

3.8 Doping control

Based on the above research together with previously published material [9], a screening method has been developed to monitor a wide range of AASs in urine from racing greyhounds. Samples are hydrolyzed with β-glucuronidase, then are purified using a simple reversed phase solid phase extraction. Extracted steroids are converted to their *O*-methyloxime and/or *tert*-butyldimethylsilyl derivatives (section 2.4.6) and are analyzed by GC-MS in multiple reaction monitoring mode. The monitored transitions for the analytes described in this article appear in Table 1.

4 Conclusions

The GC-MS methodology developed here provides sub-ng/mL sensitivity for most analytes and, combined with LC-MS coverage for stanozolol [10], has successfully been used to control the use of AASs in racing greyhounds for over 4 years. During that time, numerous positive detections have been declared for boldenone, methandriol, nandrolone and stanozolol and/or their metabolites.

Executive Summary

Boldenone undecylenate was metabolized by hydrolysis followed by oxidation of the 17β-hydroxyl, whole or partial reduction of the $\Delta^{1,4}$-3-ketone with 3α,5β- or 3β,5α-stereochemistry and hydroxylation at an unidentified site.

Danazol was converted in large part to ethisterone.

The major metabolite of ethylestrenol was not fully characterized, but appears to be a 19-norpregnanetriol.

Mesterolone was metabolized by reduction of the 3-ketone with 3β-stereochemistry, hydroxylation at an unidentified site or sites and hydroxyl oxidation.

Methandriol dipropionate was metabolized by hydrolysis and apparent conversion to 17α-methyltestosterone followed by reduction of the resulting Δ^4-3-ketone with either 3α,5β- or 3β,5α-stereochemistry and 16α-hydroxylation.

Nandrolone laurate was metabolized by hydrolysis followed by oxidation and epimerization of the 17β-hydroxyl and reduction of the Δ^4-3-ketone with 3α,5β- or 3β,5α-stereochemistry.

Norethandrolone was metabolized by reduction of the Δ^4-3-ketone with either 3α,5β- or 3β,5α-stereochemistry and 16α-hydroxylation.

Phase II β-glucuronylation of sterol metabolites is significant.

Future perspective

Future work will concentrate on expanding coverage for synthetic AASs and developing effective methods for controlling the use of endogenous AASs such as testosterone and its precursors. In the longer term, the identification of indirect markers for AAS administration or the use of longitudinal data to establish biochemical parameters for individual athletes show interesting potential for canine AAS control.

Acknowledgement

This work was financially supported by Greyhounds Australasia Ltd. and the Australian Research Council (Linkage project LP0774839).

References

1. Schanzer W, Donike M. Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Anal. Chim. Acta* 275, 23-48 (1993).

2. Reference ranges for urinary concentrations and ratios of endogenous steroids, which can be used as markers for steroid misuse, in a Caucasian population of athletes. Van Renterghem P, Van Eenoo P, Geyer H, Schänzer W, Delbeke FT. *Steroids* 75, 154-163 (2010).

3. Metabolism of anabolic steroids and their relevance to drug detection in horseracing. Teale P, Houghton E. *Bioanalysis* 2, 1085-1107 (2010).

4. Williams TM, Kind AJ, Hill DW. *In vitro* biotransformation of anabolic steroids in canines. *J. Vet. Pharmacol. Ther.* 23, 57-66 (2000).

* Describes the metabolism of testosterone, 17α-methyltestosterone, boldenone and mibolerone by canine liver microsomes.

5. Williams TM, Kind AJ, Hyde WG, Hill DW. Characterization of urinary metabolites of testosterone, methyltestosterone, mibolerone and boldenone in greyhound dogs. *J. Vet. Pharmacol. Ther.* 23, 121-129 (2000).

* Previously the most comprehensive overview of canine *in vivo* AAS metabolism available.

6. Brockwell M, Knox J, Stenhouse A, Ralston J. The identification of metabolites of testosterone, 19-nortestosterone and 1-dehydrotestosterone in greyhound urine. In: *Proceedings of the 9th International Conference of Racing Analysts and Veterinarians*. Short CR (Ed.). R&W Communications. Newmarket. UK. 1:57-68 (1992).

* The earliest published data on canine AAS metabolism in a sports doping context.

7. McKinney AR, Stewart RT, Kerwick CM, Young EB, Vadasz A, McLeod MD. The metabolism of synthetic anabolic-androgenic steroids in the greyhound: boldenone undecylenate and stanozolol. In: *Proceedings of the 17th International Conference of Racing Analysts and Veterinarians*. Houghton E, Keskin FI, Wade JF, Yazıcıoğlu N (Eds.). R&W Communications. Newmarket. UK. 116-124 (2008).

8. McKinney AR, Young EB, Cunnington K, Stewart RT, McLeod MD. The metabolism of synthetic anabolic-androgenic steroids in the greyhound: nandrolone laurate and methandriol dipropionate. In: *Proceedings of the 18th International Conference of Racing Analysts and Veterinarians*. Beresford GD, Howitt RG (Eds.). Dunmore. Auckland. New Zealand. 423-430 (2012).

9. Biddle STB, O'Donnell A, Houghton E., Creaser C. Metabolism of methyltestosterone in the greyhound. *Rapid Commun. Mass Spectrom.* 23, 713-721 (2009).

* A thorough investigation of the canine metabolism of an important AAS archetype.

10. Stewart RT, McKinney AR, Kerwick CM *et al*. Metabolism of stanozolol: chemical synthesis and identification of a major canine urinary metabolite by liquid chromatography–electrospray ionization ion trap mass spectrometry. *J. Steroid Biochem. Mol. Biol.* 117, 152-158 (2009).

** This work on stanozolol constitutes a major part of this research project.

11. Bergmann W, Kita M, Giancola DJ. Δ^1-Cholesten-3β-ol. *J. Am. Chem. Soc.* 76, 4974-4975 (1954).

12. Schänzer W, Donike M. Metabolism of boldenone in man: gas chromatographic/mass spectrometric identification of urinary excreted metabolites and determination of excretion rates. *Biol. Mass Spectrom.* 21, 3-16 (1992).

13. Mori H, Oh-uchi R. 1,4-Addition of vinylmagnesium bromide to α,β-unsaturated steroidal ketones. II. Reaction of 1-en-3-oxo steroids. *Chem. Pharm. Bull.* 23, 980–986 (1975).

14. Watabe T, Yagishita S, Hara S. 16-Oxygenated 17α-methyl-5β-androstanes. *J. Med. Chem.* 13, 311-312 (1970).

15. Organon Inc. US3026318 (A) (1962).

16. Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 906819. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

17. Hungerford NL, McKinney AR, Stenhouse AM, McLeod MD. Selective manipulation of steroid hydroxyl groups with boronate esters: efficient access to antigenic C-3 linked steroid-protein conjugates and steroid sulfate standards for drug detection. *Org. Biomol. Chem.* 4, 3951-3959 (2006).

18. McKinney AR, Suann CJ, Stenhouse AM. A stereochemical examination of the equine metabolism of 17α-methyltestosterone. *Anal. Chim. Acta* 581, 377-387 (2007).

19. McKinney AR, Ridley DD, Turner P. Equine Metabolites of norethandrolone: synthesis of a series of 19-nor-17α-pregnanediols and 19-nor-17α-pregnanetriols. *Aust. J. Chem.* 56, 829-838 (2003).

20. Tang PW, Crone DL. A new method for hydrolyzing sulfate and glucuronyl conjugates of steroids. *Anal. Biochem.* 182, 289-294 (1989).

21. Edlund PO, Bowers L, Henion J. Determination of methandrostenolone and its metabolites in equine plasma and urine by coupled-column liquid chromatography with ultraviolet detection and confirmation by tandem mass spectrometry. *J. Chromatogr.* 487, 341-356 (1989).

22. Davison C, Banks W, Fritz A. The absorption, distribution and metabolic fate of danazol in rats, monkeys and human volunteers. *Arch. Int. Pharmacodyn. Ther.* 221, 294-310 (1976).

23. Kim JY, Choi MH, Kim SJ, Kyong JB, Chung BC. GC-MS characterization of urinary metabolites and changes of ethisterone and testosterone profile after oral administration of danazol in equine. *J. Vet. Pharmacol. Ther.* 24, 147-53 (2001).

24. Ward RJ, Lawson AM, Shackleton CHL. Metabolism of anabolic steroid drugs in man and the marmoset monkey (*Callithrix jacchus*):)I. Nilevar and Orabolin. *J. Steroid Biochem.*, 8, 1057-1063 (1977).

25. Steele JW, Boux LJ, Audette RC. Anabolic steroids. Part 3: metabolism of ethylestrenol in rat liver preparations *in vitro*. *Xenobiotica* 11, 117-121 (1981).

26. Gourdie TA, Beresford GD. The detection of ethylestrenol and its metabolites in the horse. In: *Proceedings of the 10th International Conference of Racing Analysts and Veterinarians*. Kallings P, Bondesson U, Houghton E (Eds.). R&W Communications. Newmarket. UK. 214-217 (1995).

27. Kim CS, Whittem T, Beresford GD. Detection of ethylestrenol in equine urine and plasma. In: *Proceedings of the 11th International Conference of Racing Analysts and Veterinarians*. Auer DE, Houghton E (Eds.). R&W Communications. Newmarket. UK. 172-178 (1996).

28. Ho EN, Leung DK, Leung GN *et al*. Metabolic studies of mesterolone in horses. *Anal. Chim. Acta* 596, 149-155 (2007).

29. Yamada M, Aramaki S, Kurosawa M, Saito K, Nakazawa H. Detection of urinary metabolites common to structurally related 17α-alkyl anabolic steroids in horses and application to doping tests in racehorses: methandienone, methandriol, and oxymetholone. *J. Anal. Toxicol.* 32, 387-391 (2008).

30. Hungerford NL, Sortais B, Smart CG *et al*. Analysis of anabolic steroids in the horse: development of a generic ELISA for the screening of 17α-alkyl anabolic steroid metabolites. *J. Steroid Biochem. Mol. Biol.* 96, 317-334 (2005).

31. Van Puymbroeck M, Kuilman MEM, Maas RFM *et al*. 17α-Ethyl-5β-estrane-3α,17β-diol, a biological marker for the abuse of norethandrolone and ethylestrenol in slaughter cattle. *J. Chromatogr. B Biomed. Sci. Appl.*, 728, 217-232 (1999).

32. McKinney AR, Ridley DD, Suann CJ. The metabolism of norethandrolone in the horse: characterization of 16-, 20- and 21-oxygenated metabolites by GC-MS gas chromatography/mass spectrometry. *J. Mass Spectrom.*, 36, 145-150 (2001).

33. McKinney AR, Ridley DD. Synthesis of equine metabolites of anabolic steroids: Reformatsky reactions on estran-17-ones. *Aust. J. Chem.*, 54, 757-761 (2001).

34. Middleditch BS, Vouros P, Brooks CJW. Mass spectrometry in the analysis of steroid drugs and their metabolites: electron-impact-induced fragmentation of ring D. *J. Pharm. Pharmacol.*, 25, 143-149 (1973).

Table 1: MRM transitions used for AAS doping control.

Analyte	Marker for	Precursor	Transition (*m/z*)	Retention time (min)[d]
Androsta-1,4-diene-3,17-dione	Boldenone	$M^{\bullet+}$	342→311	8.44[e]
5β-Androst-1-ene-3α,17β-diol	Boldenone	$[M\text{-}t\text{Bu}^{\bullet}]^+$	461→253	9.34
5β-Androst-1-ene-3,17-dione	Boldenone	$M^{\bullet+}$	344→313	8.08
Boldenone	Boldenone	$M^{\bullet+}$	429→398	10.20
5α-Estrane-3β,17α-diol	Nandrolone	$[M\text{-}t\text{Bu}^{\bullet}]^+$	449→241	9.82
5α-Estrane-3β,17β-diol	Nandrolone	$[M\text{-}t\text{Bu}^{\bullet}]^+$	449→241	10.59
5β-Estrane-3α,17β-diol	Nandrolone	$[M\text{-}t\text{Bu}^{\bullet}]^+$	449→241	10.14
Ethisterone	Danazol	$[M\text{-Me}^{\bullet}]^+$	440→209	11.14[e]
17β-Hydroxy-5β-androst-1-en-3-one	Boldenone	$M^{\bullet+}$	431→374	9.82
Methandriol	Methandriol	$[M\text{-}t\text{Bu}^{\bullet}]^+$	475→343	12.83
1α-Methyl-5α-androstane-3β,17β-diol	Mesterolone	$[M\text{-}t\text{Bu}^{\bullet}]^+$	477→269	11.81
17α-Methyl-5α-androstane-3β,17β-diol	Methandriol, 17α-Methyltestosterone	$[M\text{-}t\text{Bu}^{\bullet}]^+$	477→269	12.97
17α-Methyl-5β-androstane-3α,16α,17β-triol	Methandriol, 17α-Methyltestosterone	$[C_{12}H_{27}Si_2O_2]^+$ [a]	259→147	16.50
Nandrolone	Nandrolone	$M^{\bullet+}$	417→360	9.73
19-Norepiandrosterone	Nandrolone	$[M\text{-}t\text{Bu}^{\bullet}]^+$	362→286	8.75
19-Noretiocholanolone	Nandrolone	$[M\text{-}t\text{Bu}^{\bullet}]^+$	362→286	8.54
19-Nor-5α,17α-pregnane-3β,17β-diol	Norethandrolone	C15/C16/C17 [b]	199→73	13.45
19-Nor-5β,17α-pregnane-3α,16α,17β-triol	Norethandrolone	$[M\text{-}t\text{Bu}^{\bullet}]^+$ [c]	493→269	17.10
Unidentified 19-norpregnanetriol	Ethylestrenol	$[C_{11}H_{23}SiO]^+$ [b]	199→73	17.00

[a] C15, C16, C17 and substituents with loss of $t\text{Bu}^{\bullet}$.

[b] D-ring fragment equivalent to those described by Middleditch *et al.* [34] for 17-trimethylsilyloxysteroids.

[c] M in this case is a bis(*tert*-butyldimethylsilyl) derivative. The free OH is presumably at C17.

[d] Temperature program: 100 °C for 1 min, 40 °C/min to 300 °C, 300 °C for 12 min. All other conditions are as in section 2.5.

[e] The *O*-methyloxime *E*- and *Z*-isomers are partly resolved.

Figure 1: Identified phase I metabolites of boldenone undecylenate.

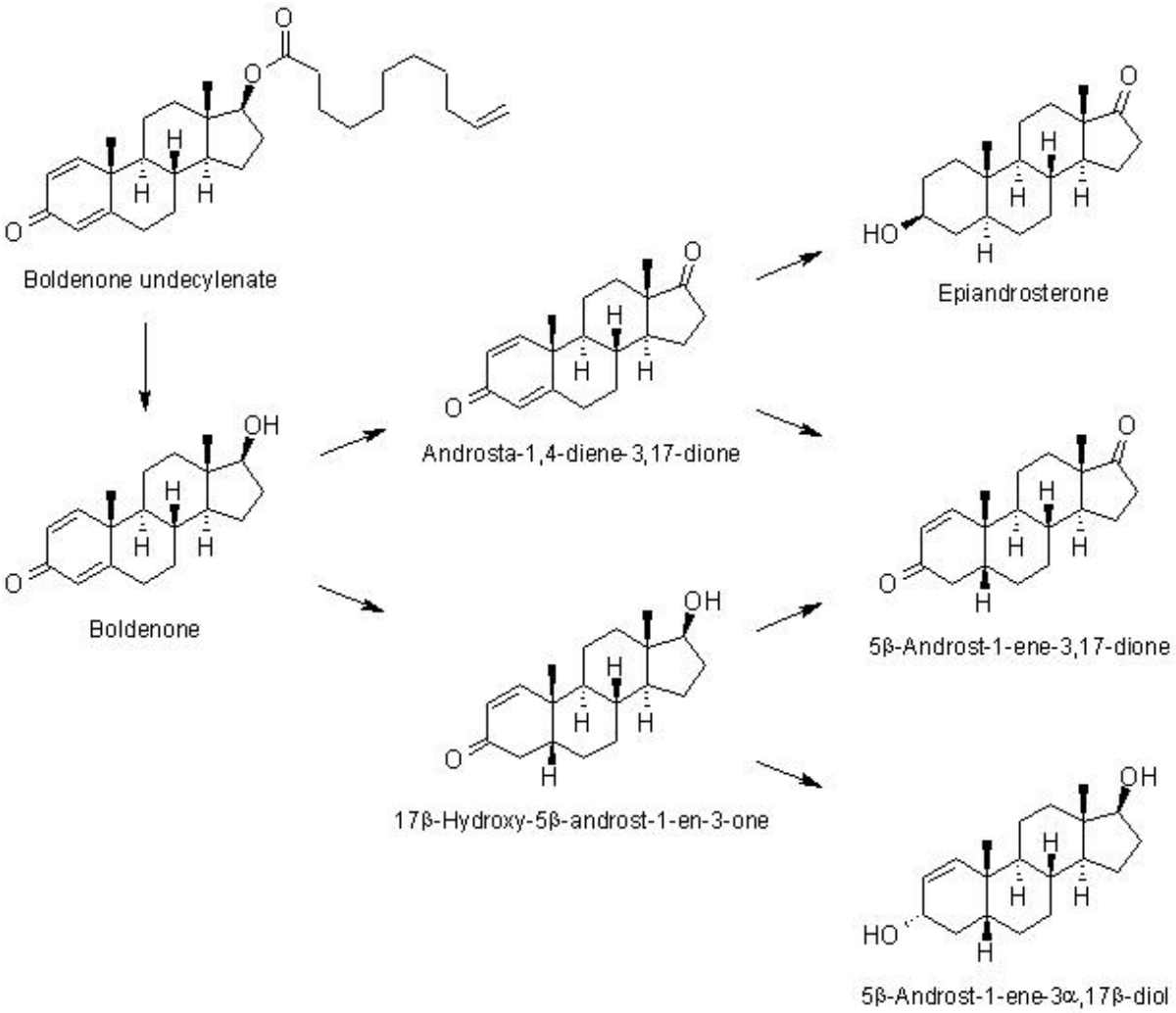


Figure 2: The major phase I metabolite of danazol.

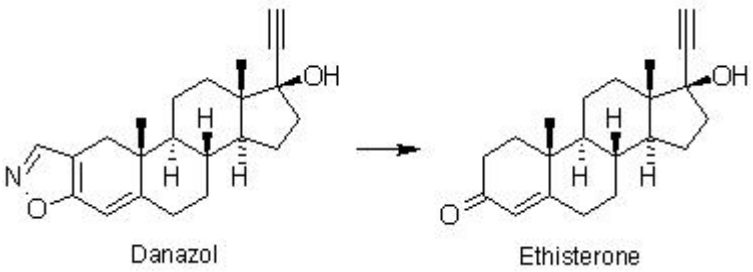
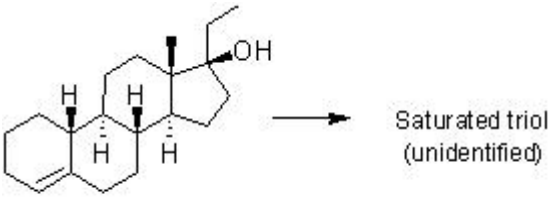


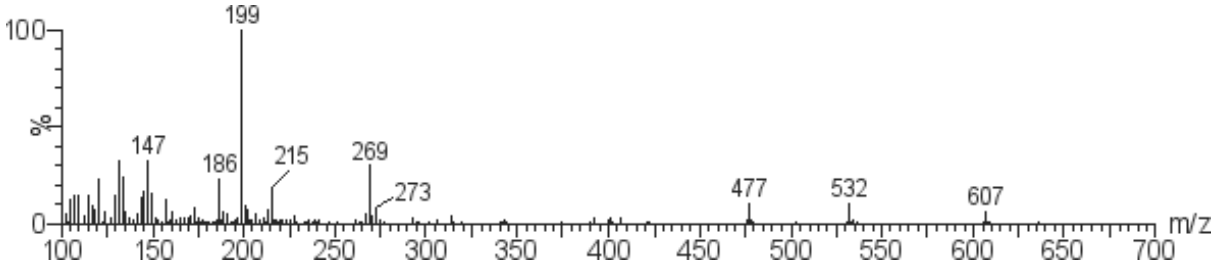
Figure 3: The major phase I metabolite of ethylestrenol.



Ethylestrenol

Figure 4: Mass spectra for the (a) tert-butyldimethylsilylated and (b) trimethylsilylated derivatives of the major ethylestrenol metabolite.

(a)



(b)

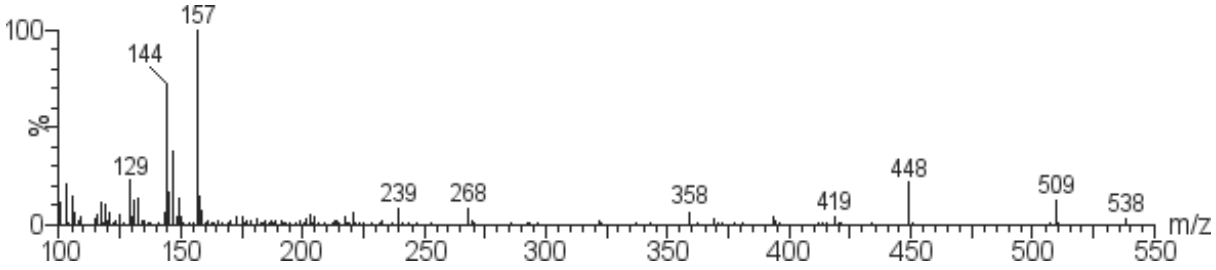
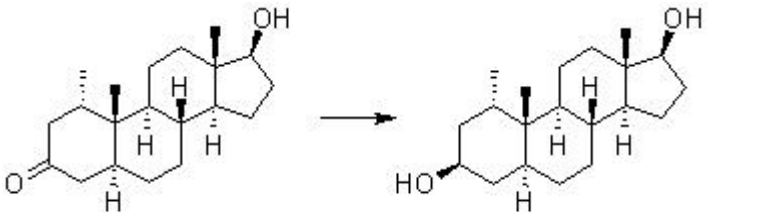


Figure 5: The major phase I metabolite of mesterolone.



Mesterolone

1α-Methyl-5α-androstane-3β,17β-diol

Figure 6: The phase I metabolism of methandriol dipropionate.

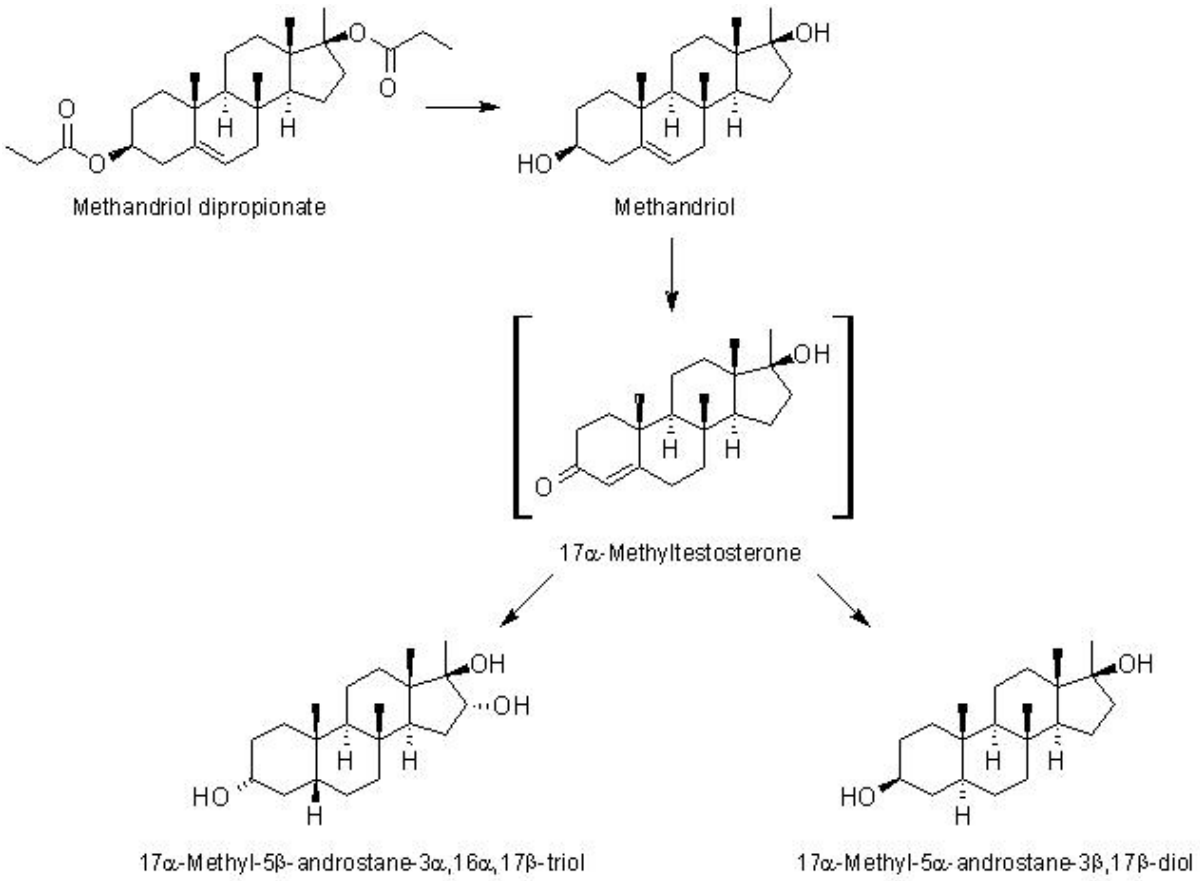


Figure 7: The phase I metabolism of nandrolone laurate.

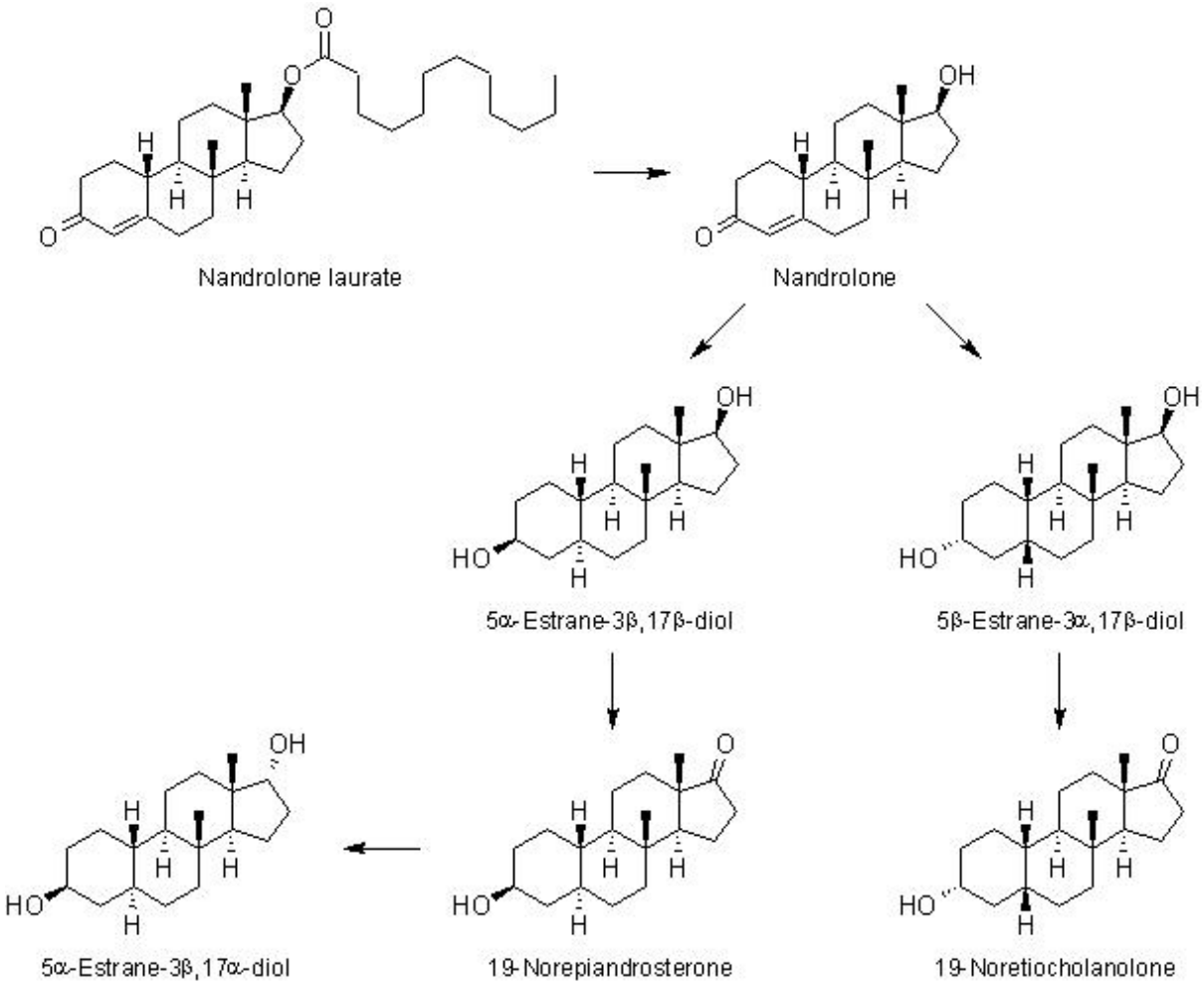


Figure 8: The identified phase I metabolites of norethandrolone.