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Metabolism of stanozolol: Chemical synthesis and identification of a major canine urinary metabolite by liquid chromatography–electrospray ionisation ion trap mass spectrometry

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ABSTRACT

The canine phase I and phase II metabolism of the synthetic anabolic-androgenic steroid stanozolol was investigated following intramuscular injection into a male greyhound. The major phase I biotransformation was hydroxylation to give 6α-hydroxystanozolol which was excreted as a glucuronide conjugate and was identified by comparison with synthetically derived reference materials. An analytical procedure was developed for the detection of this stanozolol metabolite in canine urine using solid phase extraction, enzyme hydrolysis of glucuronide conjugates and analysis by positive ion electrospray ionisation ion trap LC–MS.

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1. Introduction

Anabolic-androgenic steroids are an important class of performance enhancing drugs with potential for misuse in sport. As a result, the integrity of any sporting contest relies on stringent doping control measures targeting these agents. The detection of illicit steroid use provides significant challenges due to a range of complicating factors. Among these, the administration of an anabolic steroid frequently results in little or no excretion of the unmodified drug in the urine and instead, the steroid is converted into more hydrophilic metabolites. The detection of steroid abuse therefore requires appropriate reference materials and methods of detection for metabolites derived from steroidal agents. In the context of sporting pursuits, the metabolism of anabolic-androgenic steroids in humans [1,2] and horses [3] has been the subject of numerous detailed studies, leading to the development of a range of reference materials and robust drug screens. However, much less is known about the canine metabolism of this class of drugs [4–9].

In July 2008, Greyhounds Australasia, the regulatory entity with oversight of greyhound racing in Australia and New Zealand, extended drug testing to target all anabolic-androgenic steroids in racing greyhounds with the exception of orally administered ethylestrenol, which is approved for oestrus control in bitches. The expansion of drug testing targets requires the study of individual anabolic-androgenic steroids in greyhounds to identify the major steroidal metabolites. In some cases this requires the chemical synthesis and characterisation of new reference materials to develop and implement drug screens.

Stanozolol **1** (Fig. 1) is an anabolic-androgenic steroid with a well documented history of abuse in sport. Although the human and equine metabolism of this agent is well established [10] no study of the canine metabolism of stanozolol had been reported and so methods to test for and confirm stanozolol abuse in racing greyhounds were not rigorously established. This paper provides a full account of a study to elucidate the in vivo metabolism of the anabolic steroid stanozolol in the greyhound [11]. A structurally distinct and previously unknown major metabolite has been identified by LC–MS and confirmed by the chemical synthesis of reference materials.

2. Materials and methods

2.1. Chemicals and reagents

17α-(Methyl-$^2H_3$)-5α-androstano[3,2-*c*]pyrazol-17β-ol (stanozolol-$^2H_3$) was purchased from Sigma (Castle Hill, NSW). Boldenone-16,16,17-$^2H_3$ (boldenone-$^2H_3$), boldenone 17-β-gluc-

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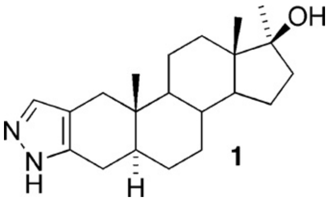
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Fig. 1. Stanozolol.

uronide potassium salt and boldenone 17-sulfate triethylammonium salt were purchased from the National Measurement Institute (Pymble, NSW). Oasis WAX solid phase extraction cartridges (3 mL, 60 mg, 60 μm) were purchased from Waters (Rydalmere, NSW). *Escherichia coli* β-glucuronidase solution (type K12) was purchased from Roche Diagnostics (Castle Hill, NSW). Anhydrous methanolic hydrogen chloride solution (1 M) was prepared according to the method of Tang and Crone [12].

2.2. Animal administration

Animal administration experiments were approved by the Queensland Department of Primary Industries and Fisheries Community Access Animal Ethics Committee. An aqueous suspension of stanozolol (Stanabolic®, Ilium, NSW, Australia; 1.4 mL = 70 mg stanozolol) was administered by intramuscular injection to one male greyhound (5 years, 35 kg). Urine samples were collected pre-administration, then at 6 h post-administration, then on days 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 21 and 29 post-administration. All samples were stored frozen until required for analysis.

2.3. Qualitative analysis

2.3.1. Sample preparation

Aliquots of urine (3 mL) were loaded onto Oasis WAX solid phase extraction cartridges which had previously been conditioned with methanol (2 mL) and water (2 mL). The cartridges were washed with sodium hydroxide solution (0.1 M; 2 mL), sodium phosphate buffer solution (0.1 M; pH 7.5; 2 mL) and water (2 mL). The cartridges were dried briefly under vacuum, then were sequentially eluted with methanol:ethyl acetate (1:1, v/v; 4 mL; free fraction), methanol:ethyl acetate:formic acid (50:50:1, v/v/v; 4 mL; β-glucuronide fraction) and methanol:ethyl acetate:diethylamine (50:50:1, v/v/v; 4 mL; sulfate fraction). All three eluates were dried by evaporation at 80 °C under a stream of nitrogen, then were individually processed as follows: (1) the free fraction was reconstituted in methanol (100 μL); (2) the β-glucuronide fraction was reconstituted in sodium citrate buffer solution (0.1 M; pH 6; 0.5 mL). *E. coli* β-glucuronidase solution (10 μL) was added and incubated overnight at 37 °C; (3) the sulfate fraction was reconstituted in anhydrous methanolic hydrogen chloride solution (1 M; 0.5 mL) and incubated for 10 min at 60 °C. To each of these fractions was then added sulfuric acid (0.35 M; 2 mL) and the acidified solutions were washed with diisopropyl ether (4 mL). The residual aqueous phases were basified with sodium hydroxide solution (2 M; 2 mL) and extracted with diisopropyl ether (4 mL). The extracts were dried by evaporation at 80 °C under a stream of nitrogen and reconstituted in acetonitrile (50 μL) and formic acid (0.1%, v/v; 50 μL) for LC–MS analysis.

2.3.2. Instrumental analysis

Qualitative LC–MS analyses were performed using a Thermo-Electron (Rydalmere, NSW) LCQ Deca XP Max ion trap mass spectrometer fed by a Surveyor pumping system equipped with a Waters XTerra C18 column (2.1 mm × 150 mm, 3.5 μm particle) and Phenomenex (Pennant Hills, NSW) Security Guard C18 guard column (4 mm × 2 mm). Sample injections (10 μL) were made into an initial mobile phase comprising 95% formic acid (0.1%, v/v) and 5% acetonitrile. The composition was held for 0.5 min, after which the acetonitrile content was increased in a linear gradient over 4.5 min to a final proportion of 95%. The final composition was held for 4 min, then was returned to the starting conditions and re-equilibrated prior to the next injection. The flow rate was 200 μL min^{-1} throughout and the column was maintained at a constant temperature of 40 °C. The MS was operated in positive ion electrospray ionisation mode with parameters optimised for 6α-hydroxystanozolol. Data were acquired in full scan product ion mode over the range *m*/*z* 90–370 using the proton adducts at *m*/*z* 329, 332, 345 and 361 as precursors for stanozolol, stanozolol-$^2H_3$, monohydroxystanozolols and dihydroxystanozolols respectively. Collision amplitudes were 45%, 45%, 42% and 42% respectively and maximum accumulation times for all experiments were 200 ms.

2.4. Quantitative analysis

2.4.1. Sample preparation

Duplicate aliquots of urine (3 mL) were prepared for quantitative analysis. Where qualitative analysis indicated a high 6α-hydroxystanozolol concentration, a second set of duplicate aliquots was prepared after a 1:10 dilution with blank canine urine (3 mL final volume). Duplicate calibrators were prepared by spiking blank canine urine (3 mL) with 6α-hydroxystanozolol at concentrations of 0, 1, 2, 4, 8, 16 and 32 ng mL^{-1}. Duplicate quality assurance samples were prepared by spiking blank canine urine (3 mL) with 6α-hydroxystanozolol (from a separate weighing to the calibrators) at a concentration of 8 ng mL^{-1}. All samples and calibrators were spiked with stanozolol-$^2H_3$ (8 ng mL^{-1}) as an internal standard, then were loaded onto conditioned Oasis WAX solid phase extraction cartridges and washed as described above. After the final wash step the cartridges were dried briefly under vacuum, then were eluted with methanol:ethyl acetate:formic acid (50:50:1, v/v/v; 3 mL). The eluates were dried by evaporation at 80 °C under a stream of nitrogen and processed to completion as described above for the β-glucuronide fraction.

2.4.2. Instrumental analysis

Quantitative LC–MS analyses were performed using an Applied Biosystems (Scoresby, VIC) API 4000 Q-Trap triple quadrupole mass spectrometer fed by a Waters Acquity UPLC system equipped with a Waters Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm particle) and Phenomenex Security Guard C18 guard column (4 mm × 2 mm). Sample injections (10 μL) were made into an initial mobile phase comprising 95% formic acid (0.1%, v/v) and 5% acetonitrile. The composition was held for 0.5 min, after which the acetonitrile content was increased in a linear gradient over 4.5 min to a final proportion of 95%. The final composition was held for 0.5 min, then was returned to the starting conditions and re-equilibrated prior to the next injection. The flow rate was 200 μL min^{-1} throughout and the column was maintained at a constant temperature of 40 °C. The MS was operated in positive ion electrospray ionisation mode with parameters optimised for 6α-hydroxystanozolol. Data were acquired in multiple reaction monitoring mode using the *m*/*z* 345 → 81 and *m*/*z* 332 → 81 transitions for 6α-hydroxystanozolol and stanozolol-$^2H_3$ respectively. Collision energies were 60 and 65 V respectively and dwell times for both experiments were 100 ms.

2.5. Analytical method validation

No thresholds are applicable to the detection of stanozolol or its metabolites in canine urine and quantitative analysis would not normally be performed in a dope testing context. The quantitative data presented here are intended as indicative only and the method has not been rigorously validated.

Analyte specificity was initially confirmed through the analysis of 10 blank canine urine samples (5 male, 5 female), none of which showed any significant matrix interference effects. Subsequently, during the routine screening of over 2000 competition samples, no evidence of matrix interference was observed. The LOD for full scan product ion qualitative analysis based on a signal to noise ratio greater than 3 was estimated as around 1 ng mL^{-1}. The LOD and LOQ for the quantitative analysis were 0.2 and 1 ng mL^{-1} respectively. The calibration curve was linear ($R = 0.995$) and the quality assurance spikes returned a mean concentration within 11% of nominal. No evidence of ion suppression effects was observed provided the analytes were extracted as bases (mixed base-neutral extracts often showed significant suppression). Analyte recoveries for the full range of conjugation states were impossible to assess directly in the absence of conjugated reference standards. However, the recovery of unconjugated 6α-hydroxystanozolol was estimated through the comparative analysis of blank canine urine samples spiked before and after the appropriate preparative sequences (using stanozolol-$^2H_3$ as an internal standard) as 80% for the solid phase extraction and 45% overall. As an indicator of likely recoveries for the phase II conjugates of 6α-hydroxystanozolol, the recoveries of boldenone 17-β-glucuronide and boldenone 17-sulfate from the solid phase extraction procedure were evaluated (using boldenone-$^2H_3$ as an internal standard) and found to be 88% and 76% respectively. Analyte recoveries in the solid phase extraction eluates other than the predicted ones were less than 1% for each phase II conjugation state.

2.6. Chemical synthesis of reference materials

2.6.1. Compound characterisation

Melting points were determined using an Optimelt Automated Melting Point System MPA 100. Optical rotations were measured using a Perkin-Elmer Polarimeter 241MC. ^{1}H nuclear magnetic resonance spectra were recorded at either 300 or 800 MHz on Mercury 300, Inova 300 or Avance 800 spectrometers at ambient probe temperatures. ^{13}C nuclear magnetic resonance spectra were recorded either 75.45 or 200 MHz on Gemini 300 or Avance 800 spectrometers with complete decoupling at ambient probe temperatures. Infra-red absorption spectra were obtained using a Perkin-Elmer Spectrum One Spectrometer. Low-resolution mass spectra were recorded on a Micromass-Waters LC-ZMD single quadrupole liquid chromatograph-MS or a VG Quattro II triple quadrupole MS instrument using electron impact techniques. High-resolution mass spectra were recorded on a VG AUTOSPEC mass spectrometer operating at 70 eV using positive ionisation. Major fragments are quoted as mass to charge ratio (assignment where possible and relative intensity). High pressure liquid chromatography (HPLC) was performed using a Waters 600E solvent delivery system with quaternary mixing 100 μL pump heads. Injections used a rheodyne 7725I manual injection valve. Sample volumes for analytical and preparative scale were 20 μL and 5 mL respectively. The detector was a Waters 2996 Photo-diode array detector. The columns were Waters Sunfire 5 micron; analytical (150 mm × 4.6 mm) and preparative (150 mm × 19 mm). The mobile phase was generated by blending of pure solvents as specified (v/v). The software was Waters Empower v.2. build 2154, service pack B. Analytical thin layer chromatography (TLC) was performed using 0.2 mm thick, aluminium-backed, pre-coated silica gel plates (Merck Silicagel 60 F_{254}). Preparative silica chromatography was performed using Merck Silicagel 60 (230–400 mesh ASTM).

2.6.2. Chemical synthesis

*2.6.2.1. 17α-Methyl-5α-androstane-3β,6α,17β-triol (**5**).* To a solution of methandriol **4** (500 mg, 1.64 mmol) in tetrahydrofuran (12 mL) cooled to −10 °C was added borane–tetrahydrofuran complex (4.10 mL, 2 M in tetrahydrofuran, 8.21 mmol) and the reaction was stirred for 3 h. Methanol (10 mL) was carefully added and a combined solution of sodium hydroxide (5 mL, 3 M) and hydrogen peroxide (15 mL, 30%, w/v) was added and the mixture stirred at room temperature overnight. The reaction was extracted into ethyl acetate (3× 20 mL), washed with brine (20 mL) and dried over magnesium sulfate. The mixture was purified by silica chromatography to provide triol **5** as a colourless solid (367 mg, 70%) A sample was recrystallised from 10% methanol:dichloromethane. mp = 216–220 °C; R_f 0.21 (80% ethyl acetate:hexane); $[\alpha]_D^{20}$ +11 (*c* 0.54, methanol); IR (thin film) 3347 (O–H), 2928, 2852 (C–H) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.58 (1H, m, *H*3), 3.42 (1H, td, *J* 10.8, 4.50 Hz, *H*6), 2.18 (1H, m, *H*4A), 2.05–1.97 (2H, m), 1.87–1.67 (4H, m), 1.65–1.17 (10H, m), 1.22 (3H, s, *Me*), 1.08–0.95 (2H, m), 0.85 (3H, s, *Me*), 0.84 (3H, s, *Me*), 0.68 (1H, m), OH not observed; ^{13}C NMR (75.45 MHz, $CDCl_3$) δ 80.9, 70.1, 68.8, 54.3, 51.9, 51.0, 54.6, 41.2, 38.2, 37.5, 36.5, 35.4, 31.9, 31.7, 30.8, 24.9, 23.1, 20.8, 13.5, 12.6. *m*/*z* (+ESI) 667 ([2M+Na]$^+$, 9), 361 ([M+K]$^+$, 100), 345 ([M+Na]$^+$, 57), 287 (79), 269 (67), 102 (64); HRMS (+ESI) calculated for $C_{20}H_{35}O_3$ ([M+H]$^+$) 323.2586, found 323.2590; (+ESI) calculated for $C_{20}H_{33}O_2$ ([M+H−H_2O]$^+$) 305.2481, found 305.2481.

*2.6.2.2. 6α,17β-Dihydroxy-17α-methyl-5α-androstan-3-one (**6**).* To a stirred solution of 17α-methyl-5α-androstane-3β,6α,17β-triol (**5**) (42 mg, 0.13 mmol) in dioxane/water/pyridine (4.0:0.22:0.021 mL) at room temperature was added freshly recrystallised *N*-bromosuccinimide (23.2 mg, 0.13 mmol) and the reaction was stirred for 5 h. A second portion of freshly recrystallised *N*-bromosuccinimide (23.2 mg, 0.13 mmol) was added and the reaction was stirred at room temperature for 24 h. The solution was acidified with concentrated hydrochloric acid to pH 3 and after 10 min, basified with sodium hydroxide (3 M) to pH 8. The organic solvent was evaporated and the aqueous phase was extracted with ethyl acetate (4× 20 mL). The combined organic extracts were dried over sodium sulfate and evaporated to dryness. The mixture was purified by silica chromatography (70% ethyl acetate:hexane) to provide ketone **6** as a colourless solid (34.8 mg, 84%). R_f 0.31 (60% ethyl acetate:hexane); $[\alpha]_D^{20}$ +22.7 (*c* 1.06, methanol) IR (thin film) 3501, 3367 (O–H), 2890 (C–H), 1650 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.47 (1H, td, *J* 10.9, 4.8 Hz, *H*6), 2.72 (1H, m, *H*4eq), 2.42–2.33 (2H, m), 2.22 (1H, t, *J* 13.9 Hz, *H*4ax), 2.06–1.97 (3H, m), 1.82–1.75 (2H, m), 1.65–1.25 (9H, m), 1.22 (3H, s, *Me*), 1.04 (3H, s, *Me*), 0.88 (3H, s, *Me*), 1.08–0.72 (2H, m), OH not observed; ^{13}C NMR (75.45 MHz, $CDCl_3$) δ 81.7, 70.0, 53.4, 53.3, 50.4, 45.7, 41.4, 39.6, 39.0, 38.7, 38.0, 36.7, 35.2, 31.6, 29.9, 26.0, 23.4, 21.1, 14.1, 12.9; *m*/*z* (+ESI) 343 ([M+Na]$^+$, 100), 80 (32); HRMS (+ESI) calculated for $C_{20}H_{32}O_3Na$ ([M+Na]$^+$) 343.2249, found 343.2254.

*2.6.2.3. 6α-Hydroxystanozolol **2**.* To a solution of 6α,17β-dihydroxy-17α-methyl-5α-androstan-3-one (**6**) (188 mg, 0.587 mmol) and ethyl formate (189 μL, 2.35 mmol) in dry tetrahydrofuran (4 mL) was added sodium hydride (188 mg, 60% dispersion in mineral oil). The reaction was heated vigorously with a heat gun to initialise the reaction and stirred at room temperature for 0.5 h. One drop of ethanol was added and the reaction stirred at room temperature for a further 0.5 h. The solution was diluted with water (15 mL) and the organic solvent was removed under reduced pressure. The residue was acidified to pH 6 with aqueous acetic acid solution (3 M) and extracted into ethyl acetate (6× 20 mL). The solution was evaporated to dryness to provide 6α,17β-dihydroxy-2-hydroxymethylidine-

17α-methyl-5α-androstan-3one as a colourless solid. This was used in the next reaction without further purification.

To a solution of 6α,17β-dihydroxy-2-hydroxymethylidine-17α-methyl-5α-androstan-3-one (assumed 0.587 mmol) in ethanol (7 mL) at 0 °C was added hydrazine monohydrate (125 μL, 2.55 mmol) and the mixture stirred at 0 °C for 1 h. The solution was diluted with water (15 mL) and the ethanol removed under reduced pressure. The aqueous phase was extracted with ethyl acetate (5× 20 mL) and the combined organic extracts were dried over sodium sulfate. The solvent was evaporated to provide the crude product. Purification by HPLC (5% methanol:dichloromethane) afforded 6α-hydroxystanozolol **2** as a colourless solid (202 mg, 77% over 2 steps, t_R = 44 min). R_f 0.17 (50% ethyl acetate:hexane); $[\alpha]_D^{20}$ +62 (*c* 2.4, methanol); IR (thin film) 3368 (O–H), 2943, 2836 (C–H), 1742 (N=C), 1653 (C=C); ^{1}H NMR (300 MHz, CD_3OD) δ 7.26 (1H, s, *H*3′), 3.49 (1H, td, *J* 10.8, 4.5 Hz, *H*6), 3.14 (1H, dd, *J* 16.6, 5.4 Hz, *H*4eq), 2.63 (1H, d, *J* 15.3 Hz, *H*1A), 2.28 (1H, dd, *J* 16.5, 11.8 Hz, *H*4ax), 2.16 (1H, d, *J* 15.0 Hz, *H*1B), 2.01 (1H, dt, *J* 12.0, 3.9 Hz, *H*7eq), 1.86 (1H, m, H16A), 1.70–1.68 (2H, m, H15A, H11B), 1.64 (1H, m, H16B), 1.60 (1H, m, H12A), 1.55 (1H, m, H8), 1.49 (1H, m, H11B), 1.47–1.37 (2H, m, H12B, H5), 1.35–1.28 (2H, m, H14, H15B), 1.21 (3H, s, *H*20), 0.95 (1H, q, *J* 11.7 Hz, *H*7ax), 0.87 (1H, m, *H*9, obscured), 0.87 (3H, s, *H*19), 0.78 (3H, s, *H*18); ^{13}C NMR (75 MHz, CD_3OD) δ [145.3 (*C*3) and 133.3 (*C*3′) determined from HMBC correlations] 115.04 (*C*2), 82.2 (*C*17), 72.0 (*C*6), 54.8 (*C*9), 51.8 (*C*14), 50.8 (*C*5), 46.7 (*C*13), 42.1 (*C*7), 39.2 (*C*10), 38.5 (*C*16), 36.5 (*C*8), 36.3 (*C*1), 32.8 (*C*12), 26.1 (*C*20), 24.4 (*C*15), 23.2 (*C*4), 21.9 (*C*11), 14.5 (*C*18), 13.1 (*C*19); *m*/*z* (+ESI) 345 ([M+H]$^+$, 100), 120 (53); HRMS (+ESI) calculated for $C_{21}H_{33}N_2O_2$ ([M+H]$^+$) 345.2542, found 345.2538.

2.6.2.4. 6β-Hydroxystanozolol **3**. To a solution of 6α-hydroxystanozolol **2** (30 mg, 0.087 mmol) in dichloromethane (2 mL) and dimethylformamide (1 mL) at room temperature was added activated 3 Å molecular sieves followed by pyridinium dichromate (49 mg, 0.13 mmol). The resulting suspension was stirred at room temperature for 14 h then quenched with diethyl ether and fil-

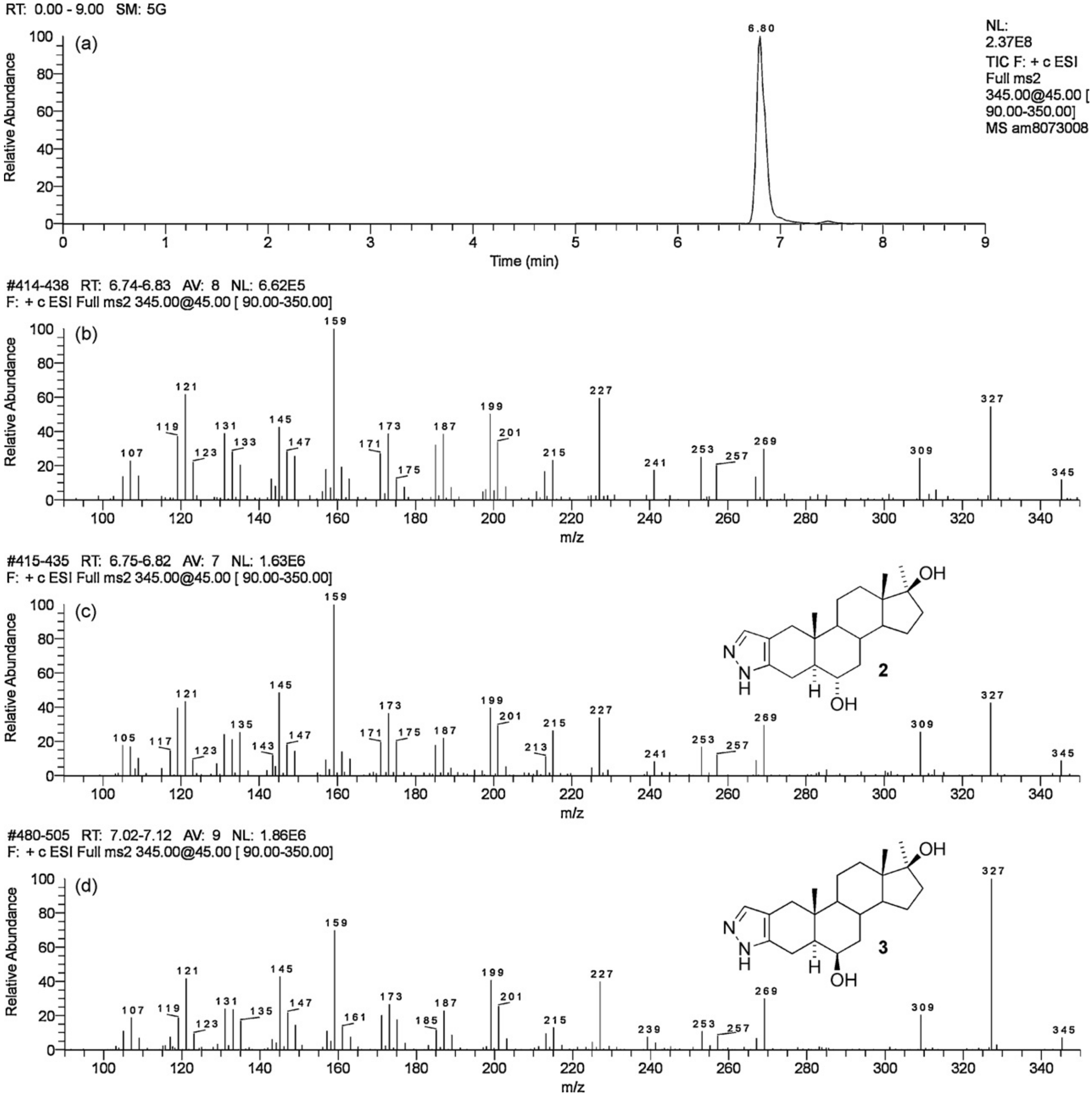
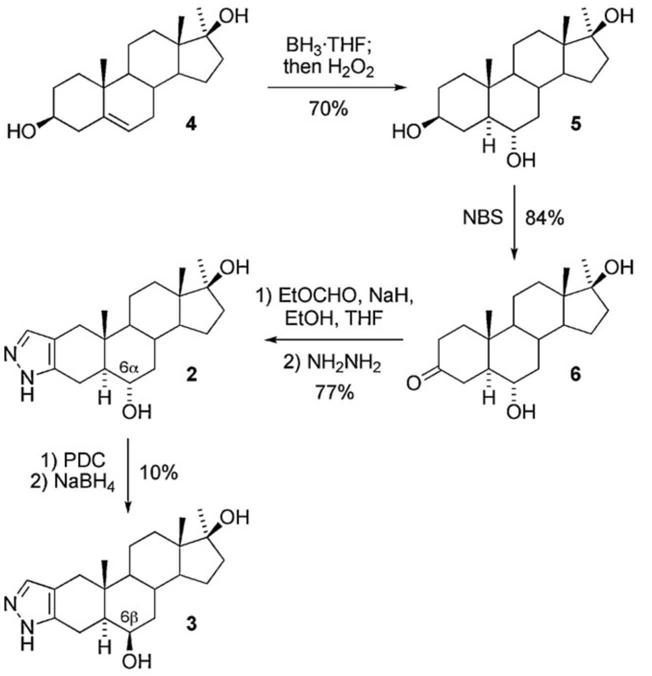


Fig. 2. (a) LC chromatogram, and (b) full scan ion trap product ion spectrum for the major canine urinary stanozolol metabolite together with corresponding spectra for, (c) 6α-hydroxystanozolol **2** and, (d) 6β-hydroxystanozolol **3**. The precursor ion in each case was *m*/*z* 345.

tered through a celite plug. The ketone product was purified via silica chromatography (10% methanol:dichloromethane) and used immediately in the next reaction.

To a stirred solution of the ketone prepared above in methanol (1 mL) at 0 °C was added sodium borohydride (3.1 mg, 0.082 mmol). The reaction was stirred at room temperature for 3 h. Water (5 mL) was added followed by aqueous hydrochloric acid (1 mL, 2 M). The mixture was extracted with ethyl acetate (7× 5 mL) and the combined organic extracts washed with brine (10 mL) and dried over magnesium sulfate. The solution was evaporated to dryness and the solid was purified by silica chromatography (10% methanol:dichloromethane) to provide 6β-hydroxystanozolol **3** as a colourless solid (3 mg, 10% over two steps). A sample was recrystallised from methanol. R_f 0.20 (10% methanol:dichloromethane); $[\alpha]_D^{20}$ +3.5 (*c* 0.75, methanol); IR (thin film) 3400 (O–H), 3060, 2925, 2862 (C–H), 1733 (N=C), 1606, 1554 (C=C) cm^{-1}; ^{1}H NMR (800 MHz, MeOD) δ 7.25 (1H, s, *H*3′), 2.92 (1H, t, *J* 14.3 Hz, *H*6), 2.56 (1H, d, *J* 14.6 Hz, *H*1A), 2.47 (1H, dd, *J* 15.4, 4.4 Hz, *H*4eq), 2.13 (1H, d, *J* 14.8, *H*1B), 1.93–1.84 (3H, m, *H*4ax, *H*7A, *H*16A), 1.71–1.65 (2H, m, *H*16B, *H*15A), 1.64–1.56 (3H, m, *H*9, *H*14, *H*15B), 1.52 (1H, m, *H*5), 1.41–1.34 (4H, m, *H*11, *H*12), 1.29 (1H, m, *H*8), 1.24–1.17 (1H, m, *H*7B, obscured), 1.21 (3H, s, *H*20), 0.95 (3H, s, *H*18), 0.91 (3H, s, *H*19); ^{13}C NMR (200 MHz, MeOD) δ 115.4 (*C*2), 82.4 (*C*17), 71.0 (*C*6), 55.5 (*C*9), 51.8 (*C*14), 49.9 (*C*5), 46.8 (*C*13), 40.6 (*C*7), 39.3 (*C*10), 37.6 (*C*16), 33.0 (*C*8), 32.4 (*C*1), 30.8 (*C*12), 26.1 (*C*20), 24.3 (*C*15), 23.8 (*C*4), 21.9 (*C*11), 15.2 (*C*18), 14.7 (*C*19), *C*3 and *C*3′ not observed; *m*/*z* (+ESI) 345 ($[\mathrm{M+H}]^+$, 100), 104 (29); HRMS (+ESI) calculated for $C_{21}H_{33}N_2O_2$ ($[\mathrm{M+H}]^+$) 345.2542, found 345.2545.



Scheme 1. Synthesis of 6-hydroxystanozolol stereoisomers.

3. Results and discussion

Stanozolol **1** is known to undergo complex metabolism in humans [13–15], cattle [16] and horses [15] with monohydroxylation at C3′, C4 and C16 being the most important metabolic pathways and the production of unidentified mono- and di-hydroxylated metabolites also observed [13–16]. In the greyhound, a major hydroxylated metabolite (t_R 6.8 min, Fig. 2a) was detected in the β-glucuronide fraction out to the day 14 sample following the intramuscular injection of an aqueous suspension of stanozolol. No retention time match was obtained with any of the commercially available hydroxylated reference materials (3′-, 4α-, 4β- and 16β-hydroxystanozolol). A very minor metabolite (t_R 7.5 min) was observed in the β-glucuronide conjugated fraction from 6 h to day 7, which was tentatively identified as 16β-hydroxystanozolol by comparison with reference materials. However subsequent experience with positive racing samples has shown 16β-hydroxystanozolol to be an unreliable marker for intramuscular stanozolol administration, so further investigation of this metabolite was not pursued. No metabolites were observed in the unconjugated or sulfate conjugated fractions, nor were stanozolol **1** itself or any di-hydroxylated metabolites detected in any fraction.

The positive ion electrospray ionisation ion trap mass spectrum of the unidentified major metabolite showed a proton adduct ($[\mathrm{M+H}]^+$ *m*/*z* 345) consistent with a hydroxystanozolol metabolite. The MS^2 spectrum of this metabolite derived from the *m*/*z* 345 precursor ion provided a more complex spectrum and showed the sequential loss of two water molecules to give fragment ions at *m*/*z* 327 and *m*/*z* 309 together with a large number of characteristic smaller fragments (Fig. 2b). The spectrum overall was extremely similar to those previously reported for various other monohydroxystanozolols under similar conditions [17–19].

Given the absence of reference materials for the canine metabolite, a comparative mass spectral analysis using available hydroxystanozolol isomers and past studies of the positive ion electrospray ionisation ion trap mass spectrometry fragmentation patterns of stanozolol [17–19] was performed to suggest the most likely hydroxylation sites and propose targets for the chemical synthesis of reference materials. This investigation suggested a 6-hydroxystanozolol as a possible identity.

In addition to the evidence derived from MS^2 fragmentation patterns, hydroxylation of the steroidal C6 position has previously been confirmed by comparison with reference materials for the canine in vitro biotransformation of both testosterone and 17α-methyltestosterone [5,6]. Furthermore 6-hydroxystanozolol has been proposed but not confirmed as a minor human metabolite of stanozolol on the basis of triple quadrupole MS^2 fragmentation patterns [15]. Given that 6-hydroxystanozolol reference materials were not commercially available and no characterisation data had been reported, we embarked on a chemical synthesis of both 6-hydroxystanozolol stereoisomers in an effort to unambiguously identify the canine metabolite.

The chemical synthesis of the 6-hydroxystanozolol isomers started from methandriol **4** (Scheme 1). Hydroboration [20] of the alkene followed by chromatographic separation of the minor 5β-androstane isomer afforded 17α-methyl-5α-androstane-3β,6α,17β-triol (**5**) in 70% yield. The stereochemistry of this intermediate was confirmed by single crystal X-ray structure determination [21]. Selective oxidation of the secondary C3-hydroxyl was achieved by means of *N*-bromosuccinimide to afford the 17α-methyl-6α,17β-dihydroxy-5α-androstan-3-one (**6**) in 84% yield [22]. This material was subjected to a two-step sequence of formylation at C2 followed by condensation with hydrazine hydrate to give 6α-hydroxystanozolol **2** (77%) [23]. Purification of the material by HPLC was performed to isolate the desired compound from a minor by-product tentatively identified as the 17-epimer. The identity of the 6α-hydroxystanozolol **2** reference material was confirmed by NMR, IR and MS data. The 300 MHz ^{1}H NMR spectrum showed a splitting pattern for the C6 oxymethine proton consistent with an axial orientation on the steroid skeleton (δ 3.49, td, *J* 10.8, 4.5 Hz). In the case of ^{13}C NMR analysis, signals corresponding to C3 and C3′ in the 1D broadband decoupled spectrum were not observed, presumably due to tautomerisation of the pyrazole ring [24]. However, the corresponding cross peaks were

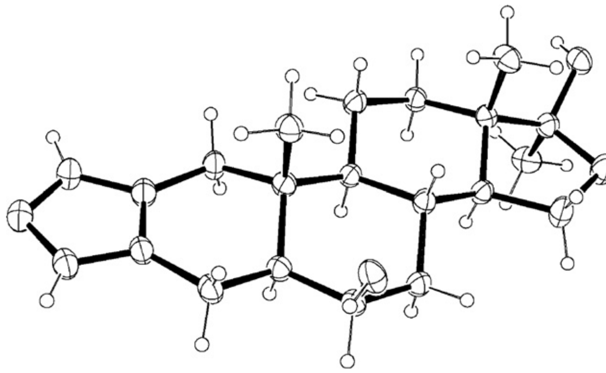


Fig. 3. Single crystal X-ray structure of 6β-hydroxystanozolol **3**.

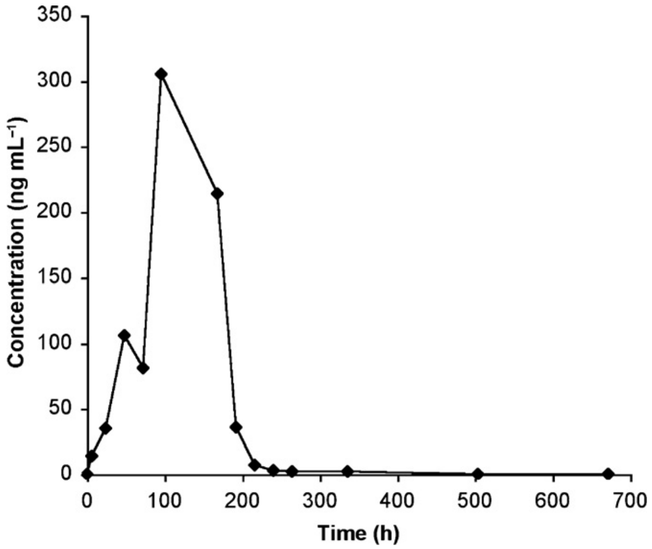


Fig. 4. Canine urinary excretion curve for 6α-hydroxystanozolol **2** following intramuscular injection of stanozolol **1** (70 mg).

observed by C–H correlation [δ 145.3 (*C*3), 133.3 (*C*3′)] in the 2D HMBC spectrum.

A two-step oxidation [25] reduction protocol was employed to convert 6α-hydroxystanozolol **2** to 6β-hydroxystanozolol **3** as a single isomer (10%). The identity of this material was confirmed by NMR, IR and MS data. Further confirmation was provided by single crystal X-ray structure determination which clearly showed the C6 hydroxyl group in the β-configuration (Fig. 3) [21].

Comparison of the canine metabolite with the synthesised reference materials 6α-hydroxystanozolol **2** and 6β-hydroxystanozolol **3** indicated a good match for the former. The 6α- and 6β-stereoisomers were resolved by liquid chromatography, and co-injection of the standards with the urine extract confirmed the co-elution of the urinary metabolite with the 6α-isomer **2**. The mass spectra were predictably similar, although a consistent difference between the two isomers was the intensity of the water loss fragment at *m*/*z* 327, which was more than twice as strong in the spectrum of the 6β-isomer **3** and appeared as the base peak. No other significant mass spectral differences were observed. The LC–MS data for the urinary metabolite and the two 6-hydroxystanozolol standards are shown in Fig. 2.

The days 1–29 urinary excretion curve for the major canine metabolite 6α-hydroxystanozolol **2** is shown in Fig. 4. Peak urinary concentrations in excess of 200 ng mL^{-1} were achieved at 4–7 days post-administration, and the analyte ceased to be detectable by day 14 with an LOD of 0.2 ng mL^{-1}.

In summary, the intramuscular administration of a registered stanozolol preparation to a male greyhound resulted in excretion of 6α-hydroxystanozolol and traces of 16β-hydroxystanozolol as β-glucuronide phase II conjugates. The excretion of 6α-hydroxystanozolol was detected from 6 h to 14 days with a peak concentration of 305 ng mL^{-1} for the therapeutic dose of stanozolol. Positive ion electrospray ionisation ion trap LC–MS provided a sensitive and specific screen for this substance in canine urine with a limit of detection of 1 ng mL^{-1} and mass spectra containing large numbers of diagnostic ions.

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