Analysis of anabolic steroids in the horse: Development of a generic ELISA for the screening of 17 α -alkyl anabolic steroid metabolites

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Abstract

Due to the potential for misuse of a wide range of anabolic steroids in horse racing, a screening test to detect multiple compounds, via a common class of metabolites, would be a valuable forensic tool. An enzyme-linked immunosorbent assay (ELISA) has been developed to detect 17 α -alkyl anabolic steroid metabolites in equine urine. 16 β -Hydroxymestanolone (16 β ,17 β -dihydroxy-17 α -methyl-5 α -androst-3-one) was synthesised in six steps from commercially available epiandrosterone (3 β -hydroxy-5 α -androst-17-one). Polyclonal antibodies were raised in sheep, employing mestanolone (17 β -hydroxy-17 α -methyl-5 α -androst-3-one) or 16 β -hydroxymestanolone conjugated to human serum albumin, via a 3-carboxymethyloxime linker, as antigens. Antibody cross-reactivities were determined by assessing the ability of a library of 54 representative steroids to competitively bind the antibodies. Antibodies raised against 16 β -hydroxymestanolone showed excellent cross-reactivities for all of the 16 β ,17 β -dihydroxy-17 α -methyl steroids analysed and an ELISA has been developed to detect these steroid metabolites. Using this 16 β -hydroxymestanolone assay, urine samples from horses administered with stanozolol (17 α -methyl-pyrazolo[4',3':2,3]-5 α -androst-17 β -ol), were analysed raw, following β -glucuronidase hydrolysis, and following solid-phase extraction (SPE) procedures. The suppressed absorbances observed were consistent with detection of the metabolite 16 β -hydroxystanozolol. Positive screening results were confirmed by comparison with standard LCMS analyses. Antibodies raised against mestanolone were also used to develop an ELISA and this was used to detect metabolites retaining the parent D-ring structure following methandriol (17 α -methyl-androst-5-ene-3 β ,17 β -diol) administration. The ELISA methods developed have application as primary screening tools for detection of new and known anabolic steroid metabolites.
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1. Introduction

Anabolic steroids are an important class of performance enhancing drug with potential for misuse in horse racing and other sports. As a result, the integrity of the horse racing industry relies on stringent doping control measures targeting these agents. Typically, the comparison of race day samples

with authentic standards by chromatographic and mass spectrometric techniques is used to screen for and confirm the presence of banned substances. However, there are a number of limitations to this approach, including the need for lengthy sample preparation prior to chromatographic analysis, and the need for authentic standards, particularly where extensive metabolism makes the detection of the parent steroid difficult or impossible. Importantly, this approach is limited to known chemical entities and may fail to detect new synthetic steroids, highlighting the need for broader screening methods [1].

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Fig. 1. The common D-ring structure which arises from the metabolism of 17-alkyl anabolic steroids.

In previous work, techniques such as immunoaffinity chromatography for sample cleanup or immunoassay for rapid primary screening, have been used to provide highly selective and sensitive recognition of steroid analytes [2–8]. A significant advantage of immunological methods is the ability of antibodies to bind broad classes of metabolites that contain a common substructure. In the main, these approaches rely on antibodies produced against antigens derived from the readily available parent drug. As a result, they are only effective in situations where there is minimal biotransformation of the parent substance [9–15].

An important class of anabolic steroid has an alkyl substituent at the 17-position, which imparts oral activity by preventing metabolic conversions of the 17-hydroxyl group, such as oxidation. However, it has been shown [16–28] that these steroids undergo extensive metabolism in horses, cattle and humans, such that little parent steroid is excreted in the urine. Commonly, phase I metabolites result from hydroxylation of the steroid, as well as A-ring reductions. Further, phase

II metabolism involves sulfate or glucuronide conjugation. Of particular relevance to equine analysis, a large number of 17-alkyl steroid phase I metabolites feature a common D-ring structure as a result of 16-hydroxylation (Fig. 1). This pattern is consistent, despite extensive variations in the remainder of the anabolic steroid skeleton, as illustrated in Fig. 2, by the 17-alkyl steroids 1–4 and examples of their corresponding metabolites 5–8.

The aim of this study was to develop a broad screening technique based on immunoassays, to detect the presence of 17-alkyl anabolic steroid metabolites in equine urine. The prevalence of 16-hydroxylated metabolites of 17 α -alkyl steroids identifies these 16,17-dihydroxylated steroids as primary targets as antigenic material for immunoassay development. 16 β -Hydroxymestanolone (9) (Fig. 3) was chosen as the initial representative 16 β ,17 β -dihydroxy-17 α -methyl steroid. Diastereoselective synthesis of 16 β -hydroxymestanolone (9) and conjugation to human serum albumin (HSA), via a (carboxymethyl)oxime tether to the A-ring, provided the antigenic material. The polyclonal antibodies raised were assessed against a panel of steroids to determine cross-reactivity and showed promise for the detection of a broad range of 16-hydroxylated D-ring steroid metabolites. Antibodies against the parent mestanolone (10) were also raised, and showed promise for the detection of metabolites retaining the non-oxidised parent D-ring structure. The enzyme-linked immunosorbent assay (ELISA)

Fig. 2. Representative 17-alkyl steroids 1–4 and examples of their corresponding metabolites 5–8.

Fig. 3. Steroids conjugated to HSA for immunoassay development.

methods developed have application as a primary screening tool for the detection of new and known steroid metabolites.

2. Materials and methods

2.1. Chemistry

2.1.1. Chemicals

Epiandrosterone (3 β -hydroxy-5 α -androst-17-one) was obtained from Steraloids (Newport, RI, USA). 17 α -Methyltestosterone (17 β -hydroxy-17 α -methyl-androst-4-en-3-one), mestanolone (17 β -hydroxy-17 α -methyl-5 α -androst-3-one), methylmagnesium bromide (3.0 M in diethyl ether), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), 1,3-dicyclohexylcarbodiimide (DCC), human serum albumin, carboxymethoxylamine hemihydrochloride, *N*-hydroxysuccinimide, dialysis tubing (25 mm $\times$ 16 mm cellulose membrane) and Dowex 50W-X8-100 ion-exchange resin were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Phosphate buffer (pH 7.5) was prepared from KH₂PO₄ solution (50 mL, 0.1 M) and sodium hydroxide solution (50 mL, 0.1 M).

2.1.2. Synthesis

2.1.2.1. General experimental. Optical rotations were measured using a PolAAR 2001 polarimeter set at the 589.3 nm sodium D line, in a 0.25 dm cell, in the solvent indicated, and at the concentration (g/100 mL) and temperature indicated. Optical rotations are quoted in 10⁻¹ deg cm² g⁻¹. Infrared spectra were recorded on a Shimadzu FTIR-8400S Fourier transform infrared spectrometer. Compounds were prepared as thin films or nujol mulls on a 0.5 cm NaCl plate, or as KBr disks, seated on a custom-made perch in the apparatus. Absorption maxima are expressed as wavenumbers (cm⁻¹). UV absorbances were recorded from 190 to 820 nm on a Hewlett-Packard 8452A diode array spectrophotometer.

¹H Nuclear magnetic resonance spectra were recorded using a Bruker Avance 200 (200.13 MHz) or a Bruker Avance 300 (300.13 MHz) spectrometer. Spectra were recorded in CDCl₃ or in CD₃OD and chemical shifts were recorded as δ values in parts per million (ppm). Signals arising from residual protio-forms of the solvent were used as internal standard (δ 7.26 and δ 3.30, respectively). Data are reported as chemical shift (δ), relative integral, multiplicity (s = singlet, br = broad, d = doublet, t = triplet and m = multiplet), assignment and coupling constant (*J* in Hz). All coupling constants and multiplicities reported are apparent values.

¹³C Nuclear magnetic resonance spectra were recorded using a Bruker Avance 200 (50.3 MHz) or a Bruker Avance 300 (75.5 MHz) spectrometer at ambient temperature with complete proton decoupling. Spectra were recorded in CDCl₃ or in CD₃OD and chemical shifts were recorded as δ values in parts per million. Signals arising from the solvent were used as internal standard (δ 77.0 and δ 49.0, respectively).

Low-resolution mass spectra were recorded by the Mass Spectrometry Unit, School of Chemistry, The University of Sydney. Major fragments are quoted as mass to charge ratio (assignment, percentage of base peak). Low-resolution mass spectra were recorded on a Finnigan Polaris Q ion trap mass spectrometer, using electron impact (+EI) ionisation mode at 70 eV or on a Finnigan LCQ ion trap mass spectrometer, using positive electrospray ionisation (+ESI) or negative electrospray ionisation (–ESI). High-resolution mass spectra were recorded on a Kratos MS25 RFA mass spectrometer, using electron impact (+EI) ionisation, operating at 70 eV, in magnetic scan, with PFK as standard (The University of Queensland), or on a Spectrospin 7T FTICR, using positive or negative electrospray ionisation (The University of New South Wales).

High performance liquid chromatography (HPLC) was carried out using a Waters system consisting of a Model M-6000A pump, a millipore model U6K injector, a model 440 ultra-violet detector (254 nm), and a R401 differential refractometer. Separation was carried out using the solvents indicated, at a flow rate of 1 mL min⁻¹, on an Alltech Altima C18 analytical column of inner diameter 4.6 mm, length 25 mm and particle size 5 μ m.

Analytical thin layer chromatography (TLC) was performed using 0.2 mm thick, aluminium-backed, precoated silica gel plates (Merck Silica gel 60 F₂₅₄). Compounds were visualised by staining with Goofy's Dip (15 g phosphomolybdic acid, 15 mL conc. sulfuric acid, 485 mL water and 2.5 g cerium sulfate) or an anisaldehyde solution (7.4 mL anisaldehyde, 383 mL ethanol 95%, 10 mL sulfuric acid and 3 mL acetic acid). Flash chromatography was performed using Merck Silica gel 60 (230–400 mesh ASTM), under a positive pressure of nitrogen, with the solvents indicated. Solvent compositions were mixed (v/v) as specified.

All solvents and reagents were purified according to standard procedures. Moisture sensitive reactions were carried out in oven-dried glassware under a dry, inert nitrogen atmosphere. Reaction temperatures were controlled using dry ice:acetone (–78 °C) or ice:water (0–5 °C) cooling baths. Concentration under reduced pressure refers to evaporation of solvent using a rotary evaporator connected to a water aspirator. Removal of residual solvent where desired, was achieved by evacuation (0.1–0.01 mmHg) with a high-stage, oil-sealed vacuum pump.

2.1.2.2. Synthesis of 16 β -hydroxymestanolone. Compound **13** was prepared in five steps, from commercially available epiandrosterone (3 β -hydroxy-5 α -androst-17-one) (**12**) according to published procedures [29–31], with only minor modifications.

2.1.2.3. 16 β ,17 β -Isopropylidenedioxy-17 α -methyl-5 α -androst-3-one (13**).** Compound **13** was synthesised according to the published procedure, from 16 β ,17 β -isopropylidenedioxy-17 α -methyl-5 α -androst-3 β -ol, and was obtained with analytical and spectral data identical to

those reported previously [29]. R_f 0.22 (ethyl acetate/hexane, 1/5); $[\alpha]_D^{22} + 16.3$ (c 0.76, chloroform), {literature [29]: $[\alpha]_D + 15.3$ (c 0.72, chloroform)}; mp: 174–178 °C (needles, acetone), (literature [29]: 173.5–176.5 °C); $\nu_{\max}$ (thin film) 2972, 2937, 2856, 1713 (C=O), 1448, 1373, 1261, 1205, 1065 cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 4.20 (1H, dd, J 7.5, 4.4 Hz, C_{16}H), 2.48–1.95 (6H, m), 1.75–0.58 (14H, m), 1.46 (3H, s, CH_3), 1.36 (3H, s, CH_3), 1.30 (3H, s, C_{20}H), 1.01 (3H, s, C_{18}H), 0.91 (3H, s, C_{19}H); ^{13}C NMR (50 MHz, CDCl_3) δ : 211.7, 112.7, 91.1, 86.2, 53.6, 52.3, 46.7, 44.8, 44.6, 38.5, 38.1, 35.7, 32.8, 31.5, 31.4, 28.7, 26.3, 25.9, 22.6, 21.1, 15.5, 11.4 (1C overlapping); HRMS (+EI): calc for $\text{C}_{23}\text{H}_{36}\text{O}_3$ (M^+) 360.2664, found 360.2662; LRMS (+EI) 360 (M^+ , 100), 345 ($[M - \text{CH}_3]^+$, 45), 285 (55), 217 (35), 127 (75).

2.1.2.4. 16 β ,17 β -Dihydroxy-17 α -methyl-5 α -androstan-3-one (16 β -hydroxymestanolone) (9). A solution of acetone 13 (440 mg, 1.2 mmol) in a mixture of methanol/distilled water (20 mL, 1/1) was stirred at room temperature under a nitrogen atmosphere. Dowex 50W-X8-100 ion-exchange resin (880 mg, 2 eq. in weight previously washed with a large amount of methanol) was added in one portion, and the resulting reaction mixture was then heated at 70 °C for 18 h. The mixture was filtered and rinsed with methanol (2 $\times$ 20 mL) to remove the resin. The mixture was concentrated to ca. 3 mL, poured onto distilled water (50 mL) and extracted into ethyl acetate (3 $\times$ 50 mL). The combined organic layers were dried (sodium sulfate) and filtered. Concentration under reduced pressure gave a colourless crude powder (400 mg). Recrystallisation in hot ethyl acetate/hexane afforded a pure mixture of two epimers **9:14** (14:1) in favour of the desired 16 β ,17 β -diol **9** (274 mg, 70%). For characterisation purposes, separation of the two epimers was achieved by HPLC (hexane/ethyl acetate, 55/45) or recrystallisation (ethyl acetate/hexane). **9**: R_f 0.2 (ethyl acetate/hexane, 1/1); $[\alpha]_D^{20} + 8.4$ (c 1.0, dichloromethane); mp: 185–186 °C; $\nu_{\max}$ (thin film) 3600–3000 (OH), 2939, 2920, 2854, 1713 (C=O), 1447, 1381, 1356, 1271, 1219, 1175, 1124, 1067, 1040, 1024 cm^{-1} ; ^1H NMR (200 MHz, CDCl_3) δ : 3.64 (1H, dd, J 7.9, 5.4 Hz, C_{16}H), 3.00–2.80 (1H, br s, OH), 2.80–2.60 (1H, br s, OH), 2.48–1.92 (6H, m), 1.78–0.63 (14H, m), 1.11 (3H, s, C_{20}H), 1.01 (3H, s, C_{18}H), 0.86 (3H, s, C_{19}H); ^{13}C NMR (50 MHz, CDCl_3) δ : 212.0, 79.1, 77.6, 53.9, 46.9, 46.7, 44.9, 44.6, 38.5, 38.1, 35.7, 35.6, 34.8, 32.3, 31.5, 28.7, 23.7, 20.8, 13.5, 11.4; HRMS (+EI): calc for $\text{C}_{20}\text{H}_{32}\text{O}_3$ (M^+) 320.2351, found 320.2350; LRMS (+EI) 320 (M^+ , 85), 232 (60), 217 (100), 159 (45).

2.1.2.5. Synthesis of steroid-HSA conjugates.

General procedure for preparation of (carboxymethyl)oximes 15–17. Carboxymethylamine hemihydrochloride (2 mmol) was added to a solution of **9–11** (1 mmol) in pyridine (10 mL). The reaction mixture was heated to 80 °C and stirred under a nitrogen atmosphere

for 1–2.5 h. The reaction mixture was poured into distilled water (100 mL) and extracted into ethyl acetate (3 $\times$ 50 mL). The combined organic layers were washed with saturated copper sulfate solution (4 $\times$ 30 mL), water (2 $\times$ 30 mL) and saturated sodium chloride solution (1 $\times$ 30 mL), and then dried (sodium sulfate) and concentrated to give the product.

2.1.2.6. 3-(Carboxymethoxyimino)-17 α -methyl-5 α -androstan-16 β ,17 β -diol (16 β -hydroxymestanolone (carboxymethyl)oxime) (15). 16 β -Hydroxymestanolone (**9**) (137 mg, 0.4 mmol) in pyridine (4 mL) was treated as per the general procedure. Flash chromatography (dichloromethane/methanol, 90/10–80/20) afforded **15** (110 mg, 65%), as colourless crystals of a 1:1 mixture of isomers. R_f 0.13 (ethyl acetate/methanol, 90/10); $[\alpha]_D^{20} + 5.2$ (c 1.0, methanol); mp: 227–228 °C; $\nu_{\max}$ (nujol) 3600–2400 (OH), 2921, 2854, 1733 (C=O), 1263, 1097, 1026, 737 cm^{-1} ; ^1H NMR (300 MHz, CD_3OD) δ : 4.50 (2H, s, OCH_2CO_2), 3.58 (1H, dd, C_{16}H , J 7.9, 5.5 Hz), 3.24, 2.99 (1H, 2 $\times$ m), 2.27–0.64 (19H, m), 1.08 (3H, s, CH_3), 0.95 (3H, s, CH_3), 0.83 (3H, s, CH_3); ^{13}C NMR (75 MHz, CD_3OD) δ : 174.2, 163.2, 79.9, 78.4, 70.7, 55.6, 55.5, 48.4, 48.0, 46.8, 46.2, 39.7, 38.5, 37.4, 37.3, 37.1, 35.9, 35.1, 33.7, 32.9, 32.8, 29.9, 29.7, 29.1, 28.4, 24.3, 22.4, 21.7, 17.7, 14.3, 13.8, 11.9, 11.7 (11C overlapping); HRMS (–ESI) calc for $\text{C}_{22}\text{H}_{34}\text{NO}_5$ ($[M - \text{H}]^-$) 392.2437, found 392.2433; LRMS (–ESI): 785 ($[2M - \text{H}]^-$, 100), 392 ($[M - \text{H}]^-$, 80).

2.1.2.7. 3-(Carboxymethoxyimino)-17 α -methyl-5 α -androstan-17 β -ol (mestanolone (carboxymethyl)oxime) (16). Commercially available mestanolone (17 β -hydroxy-17 α -methyl-5 α -androstan-3-one) (**10**) (400 mg, 1.3 mmol) in pyridine (13 mL) was treated as per the general procedure. Recrystallisation (ethyl acetate/hexane) afforded colourless, crystalline **16** [32] as a 1:1 mixture of isomers (386 mg, 78%). R_f 0.20 (dichloromethane/methanol, 90/10); $[\alpha]_D^{20} + 4.8$ (c 1.0, methanol); mp: 199–200 °C (literature [32]: 198–199 °C); $\nu_{\max}$ (nujol) 3600–2400 (OH), 1729 (C=O), 1456, 1377, 721 cm^{-1} ; ^1H NMR (300 MHz, CD_3OD) δ : 4.48 (2H, s, OCH_2CO_2), 2.98, 3.03 (1H, 2 $\times$ m), 2.25–0.54 (21H, m), 1.18 (3H, s, CH_3), 0.96 (3H, s, CH_3), 0.85 (3H, s, CH_3); ^{13}C NMR (75 MHz, CD_3OD) δ : 174.8, 163.1, 82.2, 70.8, 55.5, 52.0, 48.6, 46.8, 39.8, 39.2, 38.6, 37.6, 37.3, 35.1, 32.9, 29.9, 29.8, 26.1, 24.3, 22.4, 22.0, 14.7, 11.9, 11.7 (20C overlapping); HRMS (+ESI) calc for $\text{C}_{22}\text{H}_{36}\text{NO}_4$ ($[M + \text{H}]^+$) 378.2644, found 378.2638; LRMS (+ESI): 400 ($[M + \text{Na}]^+$, 30), 378 ($[M + \text{H}]^+$, 100), 349 (23).

2.1.2.8. 3-(Carboxymethoxyimino)-17 α -methylandrostan-17 β -ol (17 α -methyltestosterone (carboxymethyl)oxime) (17). Commercially available 17 α -methyltestosterone (17 β -hydroxy-17 α -methylandrostan-4-en-3-one) (**11**) (500 mg, 1.65 mmol) in pyridine (15 mL) was treated as per the general procedure. The residue was adsorbed onto silica from

dichloromethane/methanol (1/1) and subjected to flash chromatography (ethyl acetate/methanol, 90/10–85/15), which gave a 1:2 mixture of *Z* and *E* isomers of **17** (557 mg, 90%), as colourless crystals. R_f 0.1 (ethyl acetate/methanol, 90/10); $[\alpha]_D^{20} + 124.4$ (c 0.55, methanol); mp: 166–168 °C; $\nu_{\max}$ (KBr) 3600–2400 (OH), 2936, 1730 (C=O), 1627, 1448, 1375, 1099 cm^{-1} ; ^1H NMR (300 MHz, CD_3OD) δ : 6.45, 5.70 (1H, 2 $\times$ s, CH=), 4.53, 4.50 (2H, 2 $\times$ s, OCH_2CO_2), 3.07, 3.02 (1H, 2 $\times$ m), 2.47–2.11 (4H, m), 1.98–1.75 (4H, m), 1.70–0.76 (10H, m), 1.17, 1.15, 1.11, 0.88 (9H, 4 $\times$ s, 3 $\times$ CH_3); ^{13}C NMR (75 MHz, $\text{CD}_3\text{OD}:\text{CDCl}_3$ 3:1) δ : 173.9, 161.7, 159.0, 158.1, 155.9, 117.3, 111.8, 81.93, 81.9, 71.0, 70.8, 55.1, 54.9, 51.4, 51.3, 46.4, 46.3, 39.8, 38.9, 37.6, 37.5, 37.1, 35.5, 33.8, 33.3, 33.2, 32.9, 32.4, 26.0, 25.1, 24.0, 21.9, 21.7, 20.3, 18.4, 18.2, 14.5 (7C overlapping); HRMS (+ESI) calc for $\text{C}_{22}\text{H}_{34}\text{NO}_4$ ($[M+H]^+$) 376.2482, found 376.2478; LRMS (+ESI): 773 ($[2M+Na]^+$, 40), 398 ($[M+Na]^+$, 45), 376 ($[M+H]^+$, 100).

General procedure for synthesis of *N*-hydroxysuccinimide derivatives 18–20. *N*-Hydroxysuccinimide (1.1 mmol) was added to a solution of **15–17** (1 mmol) in dioxane (10 mL) (and dichloromethane (11 mL)). EDC (1.1 molar eq.) was added and the reaction mixture was stirred at room temperature under nitrogen, for 16–24 h. The reaction mixture was concentrated under reduced pressure. The residue obtained was partitioned between water (100 mL) and ethyl acetate (50 mL). The aqueous layer was further extracted with ethyl acetate (2 $\times$ 50 mL). The combined organic phases were washed with pH 7, buffer (50 mL) and saturated sodium chloride solution (50 mL), then dried (sodium sulfate) and concentrated.

2.1.2.9. 3-(Carboxymethoxyimino)-17 α -methyl-5 α -androstane-16 β ,17 β -diol *N*-hydroxysuccinimide ester (16 β -hydroxymestanolone (carboxymethyl)oxime *N*-hydroxysuccinimide ester) (18**).** Compound **15** (47 mg, 0.12 mmol) in dichloromethane (1.5 mL) and dioxane (1.5 mL) was treated as per the general procedure. Precipitation of the resulting solid from ethyl acetate/hexane gave **18** (40 mg, 68%) as a 1:1 mixture of isomers. R_f 0.6 (ethyl acetate); $[\alpha]_D^{20} + 8.8$ (c 0.50, chloroform); mp 88–90 °C; $\nu_{\max}$ (thin film) 3700–3000 (OH), 2937, 2856, 1823 (C=O), 1788 (C=O), 1737 (C=O), 1446, 1381, 1267, 1205, 1076 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ : 4.87 (2H, s, OCH_2CO_2), 3.63 (1H, dd, C_{16}H , J 7.9, 5.4 Hz), 3.18, 2.96 (1H, 2 $\times$ m), 2.83 (4H, s, $\text{COCH}_2\text{CH}_2\text{CO}$), 2.30–2.80 (2H, br s, 2 $\times$ OH), 2.32–0.60 (19H, m), 1.11 (3H, s, CH_3), 0.89 (3H, s, CH_3), 0.84 (3H, s, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ : 168.7, 165.7, 163.0, 162.9, 79.2, 77.7, 67.9, 54.1, 54.0, 47.0, 46.5, 45.3, 44.9, 38.2, 37.3, 36.2, 36.1, 35.6, 34.8, 33.9, 32.4, 31.6, 28.6, 28.4, 28.2, 27.4, 25.6, 23.8, 21.5, 20.6, 20.5, 13.5, 11.5, 11.3 (18C overlapping); HRMS (+EI) calc for $\text{C}_{26}\text{H}_{38}\text{N}_2\text{O}_7$ (M^+) 490.2679, found 490.2679; LRMS (+EI): 490 (M^+ , 18), 447 (35), 318 (20), 300 (12), 282 (16), 256 (20), 230 (60), 202 (35), 157 (25), 91 (100), 79 (35).

2.1.2.10. 3-(Carboxymethoxyimino)-17 α -methyl-5 α -androstane-17 β -ol *N*-hydroxysuccinimide ester (mestanolone (carboxymethyl)oxime *N*-hydroxysuccinimide ester) (19**).** Compound **16** (358 mg, 1 mmol) in dioxane (10 mL) was treated as per the general procedure using *N*-hydroxysuccinimide (218 mg, 2 mmol), except that DCC (391 mg, 2 mmol) was used instead of EDC, and the reaction mixture was filtered prior to concentration and extraction. Flash chromatography (dichloromethane/ethyl acetate, 40/60) of the oily residue afforded **19** (175 mg, 39%), as colourless crystals, and as a 1:1 mixture of isomers. R_f 0.25 (dichloromethane/methanol, 90/10); $[\alpha]_D^{20} + 6.2$ (c 1.0, dichloromethane); mp: 94–95 °C; $\nu_{\max}$ (nujol) 3700–3000 (OH), 1780 (C=O), 1708 (C=O), 1215, 1085 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ : 4.88 (2H, s, OCH_2CO_2), 2.99, 2.95 (1H, 2 $\times$ m), 2.84 (4H, s, $\text{COCH}_2\text{CH}_2\text{CO}$), 2.37–0.63 (22H, m), 1.26 (3H, s, CH_3), 0.90 (3H, s, CH_3), 0.85 (3H, s, CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ : 168.9, 165.9, 163.3, 163.2, 81.8, 68.0, 54.1, 54.0, 50.7, 49.4, 46.7, 45.7, 45.6, 39.1, 38.5, 37.5, 36.5, 36.4, 34.1, 31.8, 31.7, 28.9, 28.7, 28.4, 27.6, 26.0, 25.7, 25.1, 23.4, 21.7, 20.9, 14.1, 11.7, 11.5 (18C overlapping); HRMS (+ESI) calc for $\text{C}_{26}\text{H}_{39}\text{N}_2\text{O}_6$ ($[M+H]^+$) 475.2808, found 475.2803; LRMS (+ESI): 475 ($[M+H]^+$, 48), 453 (56), 437 (100).

2.1.2.11. 3-(Carboxymethoxyimino)-17 α -methylandrosten-17 β -ol *N*-hydroxysuccinimide ester (17 α -methyltestosterone (carboxymethyl)oxime *N*-hydroxysuccinimide ester) (20**).** Compound **17** (150 mg, 0.40 mmol) in dichloromethane (5 mL) and dioxane (4 mL) was treated as per the general procedure. Precipitation from ethyl acetate/hexane gave **20** (185 mg, 98%), as colourless crystals, and as a 1:2 mixture of *Z* and *E* isomers. R_f 0.7 (ethyl acetate); $[\alpha]_D^{20} + 105.9$ (c 0.54, chloroform); mp: 79–83 °C; $\nu_{\max}$ (KBr) 3700–3000 (OH), 2940, 2870, 1824 (C=O), 1789 (C=O), 1740 (C=O), 1630, 1448, 1431, 1376, 1205, 1079 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ : 6.38, 5.72 (1H, 2 $\times$ s, CH=), 4.89, 4.88 (2H, 2 $\times$ s, OCH_2CO_2), 3.03, 2.97 (1H, 2 $\times$ m), 2.80 (4H, s, $\text{COCH}_2\text{CH}_2\text{CO}$), 2.36–2.06 (4H, m), 1.90–0.72 (15H, m), 1.17, 1.08, 1.04, 0.85 (9H, 4 $\times$ s, 3 $\times$ CH_3); ^{13}C NMR (75 MHz, CDCl_3) δ : 168.7, 165.7, 165.6, 160.8, 158.5, 157.0, 155.5, 116.4, 110.6, 81.4, 81.39, 68.1, 68.0, 53.8, 53.6, 50.2, 50.1, 45.3, 45.2, 38.9, 38.8, 37.9, 36.5, 36.46, 35.9, 34.5, 32.9, 32.4, 32.1, 31.7, 31.4, 31.36, 25.7, 25.5, 24.3, 23.1, 20.9, 20.7, 19.4, 17.9, 17.6, 13.8 (10C overlapping); HRMS (+EI) calc for $\text{C}_{26}\text{H}_{36}\text{N}_2\text{O}_6$ (M^+) 472.2573, found 472.2567; LRMS (+EI): 472 (M^+ , 50), 457 (15), 300 (100), 282 (65), 266 (28), 242 (30), 209 (35), 157 (34), 131 (36), 120 (44), 105 (33), 91 (43), 79 (38), 77 (30).

2.1.2.12. Preparation and storage of the dialysis tubing. The commercially available dialysis tubing was treated before use. The glycerin residues were removed by washing the tubing in running water for 3–4 h. The sulfur compounds were removed by treating the tubing with a sodium sulfide

solution (0.3%, w/v) at 80 °C for 1 min. The tubing was then washed with hot distilled water (60 °C) for 2 min, followed by acidification with sulfuric acid (0.2%, v/v). Finally, the membrane was rinsed with hot distilled water to remove the acid. The resulting soft cellulose membrane can be stored several months in a solution of sodium azide (0.1%, w/v). The dialysis tubing was rinsed several times with distilled water prior to use.

General procedure for preparation of the steroid-HSA conjugates 21–23 from the carboxylic acid derivatives 15–17. EDC (0.07 mmol) in distilled water (1.0 mL) was added dropwise to carboxylic acid (**15–17**) (0.06 mmol) in dioxane (3.0 mL) and stirred for 30 min, at room temperature [33,34]. HSA (1.5 eq., w/w with carboxylic acid **15–17**) in phosphate buffer pH 7.5 (0.1 M, 3.0 mL) was added dropwise and the reaction mixture was stirred for 20 h. An additional quantity of solid EDC (0.04 mmol) was added and stirring was continued for 4 h. The reaction mixture was transferred to dialysis tubing and dialysed against stirred, distilled water (500 times the initial volume). The water was changed after 2, 4, 22, 48 and 72 h. The solution contained in the dialysis tubing was then utilised.

2.1.2.13. Human serum albumin-conjugate (21). Carboxylic acid **15** (24 mg, 0.06 mmol) was treated as per the general procedure, using dioxane (3 mL), EDC (13 mg, 0.07 mmol) in distilled water (1 mL) and HSA (36 mg, 1.5 eq., w/w) in phosphate buffer pH 7.5 (0.1 M, 3 mL). Following dialysis, the mixture was lyophilised to give conjugate **21** (43 mg), as a colourless powder.

2.1.2.14. Human serum albumin-conjugate (22). Carboxylic acid **16** (10 mg, 0.03 mmol) was treated as per the general procedure, using dioxane (2 mL), EDC (6 mg, 0.03 mmol) in distilled water (0.5 mL) and HSA (15 mg, 1.5 eq., w/w) in phosphate buffer pH 7.5 (0.1 M, 2 mL). Following dialysis, the mixture was lyophilised to give conjugate **22** (16 mg), as a colourless powder.

2.1.2.15. Human serum albumin-conjugate (23). Carboxylic acid **17** (24 mg, 0.064 mmol) was treated as per the general procedure, using dioxane (3 mL), EDC (13.5 mg, 0.070 mmol) in distilled water (1.0 mL) and HSA (36 mg, 1.5 eq., w/w) in phosphate buffer pH 7.5 (0.1 M, 3.0 mL). The solution contained in the dialysis tubing was then adjusted to pH 4.5 with 1 M HCl solution (two drops). A colourless precipitate was observed and after the addition of acetone (100 μ L) and solid sodium chloride (2 mg), the mixture was centrifuged (2000 $\times$ g for 20 min). The supernatant was collected and the precipitate was resuspended in distilled water. Acetone (100 μ L) and solid sodium chloride (2 mg) were added, and the centrifugation process was repeated twice. The precipitate obtained was redissolved in water (2.0 mL) by the addition of a minimal quantity of saturated sodium hydrogencarbonate solution. The resulting clear

solution was lyophilised to give **23a**, as a colourless powder (13 mg). Lyophilisation of the combined supernatants afforded **23b** (46 mg) as a colourless powder.

General procedure for preparation of the steroid-HSA conjugates 24–26 from the N-hydroxysuccinimide ester derivatives 18–20. HSA (2.5 eq., w/w) was dissolved in phosphate buffer pH 7.5 (0.05 M, 1.0 mL) and cooled to 4 °C [34,35]. N-Hydroxysuccinimide ester derivative (**18–20**) (0.05 mmol) in DMF (1.5 mL) was added dropwise. The reaction mixture was stirred between 2 and 7 °C for 12–24 h. The reaction mixture was transferred to dialysis tubing and dialysed against stirred, distilled water (500 times the initial volume). The water was changed after 2, 4, 22, 48 and 72 h. The solution contained in the dialysis tubing was then adjusted to pH 4.5, with 1 M HCl solution (2–3 drops). A colourless precipitate was observed, and after the addition of acetone (100 μ L) and sodium chloride (2 mg), the mixture was centrifuged (3600 $\times$ g for 20 min). The supernatant was removed and the precipitate resuspended in distilled water. Acetone (100 μ L) plus solid sodium chloride (2 mg) were added and the centrifugation process repeated twice. The precipitate obtained was redissolved in water (2.0 mL) by the addition of a minimal quantity of saturated sodium hydrogencarbonate solution. The resulting clear solution was lyophilised to give **24–26**, as a colourless powder.

2.1.2.16. Human serum albumin-conjugate (24). N-Hydroxysuccinimide ester derivative **18** (22 mg, 0.05 mmol) was treated by the general procedure to afford the conjugate **24** (44 mg), as a colourless powder.

2.1.2.17. Human serum albumin-conjugate (25). N-Hydroxysuccinimide ester derivative **19** (15 mg, 0.04 mmol) was treated by the general procedure to afford the conjugate **25** (55 mg), as a colourless powder.

2.1.2.18. Human serum albumin-conjugate (26). N-Hydroxysuccinimide ester **20** (0.023 g, 0.048 mmol) was treated as per the general procedure to give **26a**, as a colourless powder (42 mg). Lyophilisation of the combined supernatants from the centrifugation afforded **26b** (12 mg), as a colourless powder.

2.1.3. Determination of steroid/HSA molar ratio

The extinction coefficient (ϵ) for 17 α -methyltestosterone (carboxymethyl)oxime (**17**) was calculated from UV absorbance measurements at λ_{max} 254 nm. The blank solution consisted of 10% EtOH/0.1 M pH 7.5 buffer solution. Concentrations of compound **17** of 1.27×10^{-4} , 6.34×10^{-5} , 3.17×10^{-5} and 1.27×10^{-5} mol L $^{-1}$ in 10% EtOH/0.1 M pH 7.5 buffer solutions were employed and absorbances at λ_{max} 254 were measured as 1.952, 1.023, 0.5717 and 0.2295, respectively. From a plot of absorbance versus concentration, ϵ for compound **17** was determined to be 15,680 L mol $^{-1}$. Steroid/HSA molar ratio calculations assumed that ϵ (compound **17**) = ϵ (conjugated steroids).

Table 1
Molar ratios (steroid/HSA) determined by UV analyses at 254 nm

Conjugate	Molar ratio (steroid-HSA)
23a	12
23b	9
26a	28
26b	N.D.

For the determination of the steroid/HSA molar ratio, UV absorbances were measured at λ 254 nm. The blank consisted of 0.1 M pH 7.5 buffer solution. Absorbances at λ 254 nm were determined for dialysed HSA and conjugates **23a**, **23b**, **26a** and **26b** as 0.1 M pH 7.5 buffer solutions, each at a concentration of 1.0×10^{-6} M in 50 mL (the molecular weight of the conjugates was assumed to be 76,101 g mol⁻¹). The contribution to the absorbance at 254 nm of the steroid residues in each of the conjugates was obtained by subtracting the absorbance of HSA (at 254 nm) from that of the conjugates. The molar ratio was then determined using the method of calculation described by Erlanger et al. [36] and using a molecular weight of HSA of 66,462 g mol⁻¹. In the case of **26b**, much lower absorbances at λ 208 nm (λ_{max} of HSA) and λ 254 nm were obtained, and so the molar ratio was not determined for this fraction. The molar ratios for **23a**, **23b** and **26a** are presented in Table 1.

2.2. Production of polyclonal antibodies

All procedures that involved the use of animals were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 1997, Australian Government Publishing Services, Canberra, ACT), and were approved by the Animal Care and Ethics Committee of Charles Sturt University.

Sixteen Merino wether lambs (35–45 kg) were purchased from a commercial breeder and transported to Charles Sturt University, Wagga Wagga, where they were kept on pasture with ad libitum access to feed and water. Individual sheep were identified by fitting ear tags and were treated for internal parasites using ivermectin. On the first day of vaccination, a 10 mL blood sample was collected from each animal by jugular venipuncture. Then, each lamb was given a subcutaneous injection of 2 mL of vaccine, divided over four sites around the neck and shoulders to minimise the severity of any site reaction. The vaccine comprised steroid-HSA conjugate **21**, **22**, **24** or **25** (500 μ g) dissolved in sterile isotonic saline (1 mL), and emulsified with Freund's complete adjuvant (1 mL) (Sigma Chemical, St. Louis MO, US). Each of the four steroid conjugates **21**, **22**, **24** and **25** were administered to four lambs.

Twenty-eight and 56 days after the primary vaccination, the sheep each received a 2 mL booster vaccination containing steroid-HSA conjugate **21**, **22**, **24** or **25** (500 μ g) emulsified with Freund's incomplete adjuvant (1 mL) (Sigma Chem-

ical, St. Louis MO, US). On day 87, the sheep were exsanguinated, the blood was centrifuged (3000 $\times$ g for 20 min), and the serum stored frozen at -20°C , until the determination of sera antibody titres (Section 2.3.3) and cross-reactivity (Section 2.3.4) by ELISA. Sera were then transported from Wagga Wagga to Sydney, and stored at 4°C .

2.3. Biochemistry

2.3.1. Chemicals and reagents

The following buffers were used in the assay: *carbonate coating buffer* (0.1 M, pH 9.6), sodium carbonate (0.795 g), sodium hydrogencarbonate (1.46 g), distilled water (500 mL); *wash buffer*, sodium chloride (17 g), Tween 20 (1.1 g), distilled water (2.0 L); *assay buffer*, phosphate buffered saline tablets (two tablets), Tween 20 (0.22 g), distilled water (400 mL) and *stop solution* (0.2 M H₂SO₄).

All buffers used Milli Q Water except the wash buffer, which used Milli Ro; sodium carbonate (anhydrous) and sodium bicarbonate powders were obtained from Mallinckrodt Speciality Chemicals; sodium chloride was obtained from Selby-Biolab; and polyoxyethylenesorbitan monolaurate (Tween 20), phosphate buffered saline tablets, donkey anti-sheep IgG (whole molecule) peroxidase conjugate (antibody 2) and ovalbumin were from Sigma-Aldrich (Castle Hill, NSW, Australia). Liquid substrate for horseradish peroxidase (HRP), 3,3',5,5'-tetramethylbenzidine (TMB), was from TCC. Microtitre plates were clear, non-sterile, flat-bottomed plates with 96 wells from both Greiner Bio-one and ICN Biomedicals. An *Helix pomatia* β -glucuronidase preparation from Biozyme Laboratories was employed, P/N GH2G, 8.977×10^6 units per 100 mL. Anhydrous methanolic HCl (1 M) was prepared according to the method of Tang and Crone [37]. Methandriol (17 α -methylandroster-5-ene-3 β ,17 β -diol) was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). The preparation of 19-nor-5 α ,17 α -pregnane-3 β ,17 β -diol (GCMS internal standard) has been described previously [22].

2.3.2. Instrumentation

Absolut Nexus solid-phase extraction (SPE) cartridges (30 mg, 1 mL) were obtained from Varian (Mulgrave, VIC., Australia); Oasis MCX SPE cartridges (30 mg, 1 mL) were from Waters (Rydalmere, NSW, Australia). Solid-phase extractions were automated using a Gilson (Villiers-le-Bel, France) Aspec XL4 liquid-handling system. ELISA work employed a Bio-Tek Instruments (Winooski, VT, USA). Model ELP 35 microplate washer and a Sorin Biomedica (Saluggia, Italy) Eti-System microplate reader. Absorbances were measured at the dual wavelengths of 450 and 620 nm. GCMS data were obtained in EI mode on a Shimadzu QP-5000 instrument equipped with an AS-1400 autosampler. The column was a SGE BPX-5 capillary column (12 m $\times$ 0.22 mm i.d., 0.33 μ m film thickness) with helium as carrier gas.

2.3.3. Determination of antibody titres by ELISA to optimise sera concentrations

2.3.3.1. Conjugation of antigens and immobilisation (plate coating). To carboxylic acids **15** or **16** (20 mg) in 1,4-dioxane (2.5 mL) was added a solution of EDC (10 mg) in deionised water (1 mL). After stirring for 30 min, a solution of ovalbumin (30 mg) in pH 7.5 sodium phosphate buffer (0.1 M, 4 mL) was added and the reaction mixture was stirred for 20 h. A further quantity of EDC (5 mg) was then added and the reaction was stirred for 4 h. The reaction mixture was transferred to dialysis tubing and dialysed against stirred, deionised water for 3 days. The contents of the dialysis sack were centrifuged to remove a colourless precipitate, and the supernatant fraction was lyophilised to give ovalbumin conjugates of **15** (5.1 mg) or **16** (4.1 mg).

Microtitre plate wells were coated with the appropriate antigen, either ovalbumin conjugated to **15** or **16** ($0.5 \mu\text{g}$, $5 \mu\text{g mL}^{-1}$), in pH 9.6 carbonate coating buffer (0.1 M, $100 \mu\text{L}$) and incubated overnight at 4°C . The plates were then washed six times with wash buffer and tapped dry to give *16 β -hydroxymestanolone plates* or *mestanolone plates*, respectively. Coated plates were either used immediately, or stored for up to two weeks at 4°C , wrapped in the presence of desiccant.

2.3.3.2. Procedure for 16 β -hydroxymestanolone plates with antisera produced against **21 and **24** and for mestanolone plates with antisera produced against **22** and **25**.** Antisera raised in the sheep against conjugates **21**, **22**, **24** or **25** were each diluted in assay buffer to the first appropriate level, then serially diluted, and $100 \mu\text{L}$ of each dilution was placed in a separate well of a prepared plate (Section 2.3.3). The plate was then incubated at 37°C for 60 min. The plate was then washed six times with wash buffer, and tapped dry. Antibody 2 (donkey anti-sheep conjugate) diluted (1/2500) in assay buffer ($100 \mu\text{L}$) was then added to each well, and the plate was incubated at 37°C for 60 min.

The plate was then washed six times with wash buffer and tapped dry. The HRP–TMB substrate ($100 \mu\text{L}$) was added and incubated for 20 min, at room temperature. Stop solution ($100 \mu\text{L}$, $0.2 \text{ M H}_2\text{SO}_4$) was then added to each well, and the plate was read at the dual wavelengths of 450 and 620 nm. Serum collected from each sheep on day 0 (before vaccination) was used to determine control absorbance levels. Non-specific binding was relatively low in this assay and so a blocking reagent was not necessary. Antibody titres were determined for day 87 antisera, and are listed in Table 2. The best antibody titres for each treatment were determined using an absorbance ($B_{\text{DAY } 87}$) cut-off level of 0.2 absorbance units above the control absorbance ($B_{\text{DAY } 0}$).

As determined with the 16 β -hydroxymestanolone plate, sera from sheep vaccinated with conjugate **24** gave higher antibody titres than sera from sheep given conjugate **21**. With the mestanolone plate, sera arising from treatment with vaccine containing conjugate **25** gave higher antibody titres than treatment with vaccine containing conjugate **22**. For use in

Table 2

Antibody titres determined for four HSA conjugates 87 days after primary vaccination

Antigen	Sheep	Titre (in which $B_{\text{DAY } 87} - B_{\text{DAY } 0} \geq 0.2$)
21	A ₂₁	1/204800
	B ₂₁	1/204800
	C ₂₁	1/204800
	D ₂₁	1/204800
22	A ₂₂	1/51200
	B ₂₂	1/25600
	C ₂₂	1/102400
	D ₂₂	1/204800
24	A₂₄	1/1638400
	B ₂₄	1/1638400
	C ₂₄	1/819200
	D ₂₄	1/819200
25	A ₂₅	1/1638400
	B₂₅	1/3276800
	C ₂₅	1/1638400
	D ₂₅	1/1638400

the ELISA, the highest titre antisera produced against **24** (1/1638400, sheep A₂₄) and against **25** (1/3276800, sheep B₂₅) were chosen and both were found to be adequate for use at a 1/819200 dilution in assay buffer.

2.3.4. Determination of cross-reactivities by ELISA

2.3.4.1. Procedure for 16 β -hydroxymestanolone plates with antisera produced against **24 and for mestanolone plates with antisera produced against **25**.** Anabolic steroid standards (refer to Tables 3 and 4) were prepared in assay buffer at a concentration of 100 ng mL^{-1} , as well as 16 β -hydroxymestanolone at 1.6, 3.2, 6.3, 12.5, 25, 50, 100 and 200 ng mL^{-1} , and $50 \mu\text{L}$ was added to each well, followed by $50 \mu\text{L}$ 1/819200 diluted sheep antiserum. The plate was then shaken, covered, and incubated at 37°C for 60 min. The plate was then washed six times with wash buffer, and dried.

Antibody 2 (donkey anti-sheep conjugate) was then diluted 1/2500 in assay buffer and $100 \mu\text{L}$ was added to each well. The reagents were mixed by gently tapping the plate before it was wrapped and incubated at 37°C for 60 min. The plate was then washed six times with wash buffer, tapped dry, and then the HRP–TMB substrate ($100 \mu\text{L}$) was added and incubated for 20 min, at room temperature. Next, stop solution ($100 \mu\text{L}$, $0.2 \text{ M H}_2\text{SO}_4$) was added to each well, and the plates were read at the dual wavelengths of 450 and 620 nm. $B/B_0\%$ absorbances were then measured, and cross-reactivities were determined, as shown in Table 3 (16 β -hydroxymestanolone plates) and Table 4 (mestanolone plates).

2.3.4.2. Validity of the cross-reactivity studies. The $B/B_0\%$ absorbances for each ligand were determined by the same ELISA assay for a number of the cross-reactive anabolic steroid standards, at various concentrations, for comparison with the original steroid antigen, 16 β -hydroxymestanolone (for the 16 β -hydroxymestanolone plate) or mestanolone

Table 3

Percentage cross-reactivities for anabolic steroids standards: 16 β -hydroxymestanolone plate with antibodies produced against human serum albumin-conjugate (24)

Anabolic steroid standard	Cross-reactivity (%)
16 β -Hydroxymestanolone	100
16 β -Hydroxystanozolol	100
17 α -Methyl-5 α -androstane-3 β ,16 β ,17 β -triol	100
16 β ,17 β -Dihydroxy-17 α -methyl-5 α -androst-1-en-3-one	100
16 β -Hydroxymethandrostenolone	84.3
16 α -Hydroxyfurazabol	39.8
19-Nor-5 α ,17 α -pregnane-3 β ,16 β ,17 β -triol	5.5
17 α -Hydroxyprogesterone	2.8
Norbolethane	2.8
Furazabol	2.4
5 α -Estrane-3 β ,17 α -diol	2.4
6 β -Hydroxymethandrostenolone	2.3
Stanolone	2.2
Epitestosterone	2.2
Trenbolone	2.2
Ethylestrenol	2.1
Tetrahydrogestrinone (THG)	2.0
Fluoxymesterone	2.0
Oxymetholone	2.0
3'-Hydroxystanozolol	2.0
Boldenone	1.8
Testosterone	1.8
5 α -Estrane-3 β ,16 α ,17 β -triol	1.8
5 α -Estrane-3 β ,16 β ,17 β -triol	1.8
17 α -Methyl-5 α -androstane-3 α ,17 β -diol	1.7
17 β -Estradiol	1.6
Estrone	1.6
Formebolone	1.6
Epitenbolone	1.6
Ethinylestradiol	1.6
Hydrocortisone	1.6
Nandrolone	1.5
Ethisterone	1.5
Mibolerone	1.4
Norpregnandiol	1.4
Androsterone	1.4
Bolasterone	1.3
17 α -Estradiol	1.3
Altrenogest	1.3
Progesterone	1.2
Methyltestosterone	1.2
17 α -Methyl-5 α -androstane-3 β ,17 β -diol	1.2
Normethandrone	1.2
Mestanolone	1.2
Methandriol	1.1
4 α -Hydroxystanozolol	1.1
Danazol	1.1
Oxymesterone	1.1
17 α -Methyl-5 α -androstane-3 β ,16 α ,17 β -triol	1.1
Oxandrolone	1.1
Dehydroandrosterone	1.0
4 β -Hydroxystanozolol	<1
Methandrostenolone	<1
Stanozolol	<1
19-Nor-5 α ,17 α -pregnane-3 β ,16 α ,17 β -triol	<1
Norethandrolone	<1

Table 4

Percentage cross-reactivities for anabolic steroids standards: mestanolone plate with antibodies produced against human serum albumin-conjugate (25)

Anabolic steroid standard	Cross-reactivity (%)
Mestanolone	100
Methyltestosterone	100
17 α -Methyl-5 α -androstane-3 β ,17 β -diol	100
Methandriol	78.7
Oxymetholone	60.3
Oxymesterone	41.8
Furazabol	39.3
Stanozolol	39.3
Ethisterone	34.4
Oxandrolone	33.5
Normethandrone	29.9
Methandrostenolone	28.1
17 α -Methyl-5 α -androstane-3 α ,17 β -diol	27.8
4 α -Hydroxystanozolol	26.2
Bolasterone	25.2
Mibolerone	24.9
4 β -Hydroxystanozolol	23.2
3'-Hydroxystanozolol	17.1
Danazol	16.5
Norpregnandiol	9.1
Ethinylestradiol	9.1
Norethandrolone	8.4
Stanolone	6.3
Testosterone	5.6
17 α -Methyl-5 α -androstane-3 β ,16 β ,17 β -triol	5.6
16 β -Hydroxymestanolone	5.4
Norbolethane	4.9
16 β -Hydroxystanozolol	4.7
Tetrahydrogestrinone (THG)	4.1
16 β ,17 β -Dihydroxy-17 α -methyl-5 α -androst-1-en-3-one	3.3
16 β -Hydroxymethandrenone	3.0
16 α -Hydroxyfurazabol	2.8
6 β -Hydroxymethandrostenolone	1.9
Dehydroandrosterone	1.8
19-Nor-5 α ,17 α -pregnane-3 β ,16 α ,17 β -triol	1.7
Nandrolone	1.6
Fluoxymesterone	1.3
Androsterone	1.3
Boldenone	1.2
Ethylestrenol	1.1
Progesterone	<1
Altrenogest	<1
Trenbolone	<1
17 α -Methyl-5 α -androstane-3 β ,16 α ,17 β -triol	<1
19-Nor-5 α ,17 α -pregnane-3 β ,16 β ,17 β -triol	<1
17 β -Estradiol	<1
17 α -Estradiol	<1
Estrone	<1
Epitestosterone	<1
Formebolone	<1
Epitenbolone	<1
17 α -Hydroxyprogesterone	<1
Hydrocortisone	<1
5 α -Estrane-3 β ,17 α -diol	<1
5 α -Estrane-3 β ,16 α ,17 β -triol	<1

Fig. 4. Validation of the cross-reactivity comparisons: parallelism of various cross-reacting steroid ligands on the 16 β -hydroxymestanolone plate. [Parent compound is 16 β -hydroxymestanolone (9). 100% = 16 β -hydroxystanozolol, 84% = 16 β -hydroxymethandrenone, 40% = 16 α -hydroxyfurazabol, 5.5% = 19-nor-5 α ,17 β -pregnane-3 β ,16 β ,17 β -triol and 0.963% = stanozolol].

(for the mestanolone plate). Plots of percentage absorbance ($B/B_0\%$) versus concentration were constructed, as shown in Figs. 4 and 5. The parallel nature of these plots, relative to the original steroid, validates the cross-reactivity comparisons, which imply that cross-reactive steroids bind to the antibodies with similar affinities.

2.3.5. Equine administration

Both animal administration trials were approved by the New South Wales Thoroughbred Racing Board Animal Care and Ethics Committee.

An aqueous suspension of stanozolol (4) (Stanazol[®], RWR Veterinary Products, Glenorie, NSW, Australia; 5 mL = 250 mg stanozolol) was administered to a thoroughbred gelding (7 years; 580 kg) by intramuscular injection. Urine samples were collected daily, for 28 days, by con-

Fig. 5. Validation of the cross-reactivity comparisons: parallelism of various cross-reacting steroid ligands on the mestanolone plate. [Parent compound is mestanolone (10). 100% = methandriol, 60.3% = oxymetholone, 34.4% = ethisterone, 16.5% = danazol and 0.631% = estrone].

ditioned spontaneous voiding and immediately frozen and stored at -20°C until required for analysis.

An oil-based solution of methandriol dipropionate (Protabol[®], RWR Veterinary Products, Glenorie, NSW, Australia; 5 mL = 375 mg methandriol dipropionate) was administered to a standardbred gelding (aged; 560 kg) by intramuscular injection. Urine samples were collected daily, for 28 days, by conditioned spontaneous voiding and immediately frozen and stored at -20°C until required for analysis.

2.3.6. ELISA development: application to post-administration equine urine samples

2.3.6.1. Analysis of 16 β -hydroxymestanolone assay with equine urine following stanozolol (4) administration. ELISA plates were prepared as in Section 2.3.3 and the assay was performed, as per the method described in Section 2.3.4, using 50 μL of test sample per well. The 1.6 ng mL^{-1} 16 β -hydroxymestanolone standard showed clearly suppressed binding compared to a reagent blank. All administration samples were analysed with this procedure, varying the standards, antibodies and plates as appropriate.

Negative results (lack of detectable suppression of bindings) were obtained with raw post-administration urine samples using the 16 β -hydroxymestanolone assay. Raw urine was hydrolysed overnight at 37°C with 1/100 dilution of β -glucuronidase, and examined by the 16 β -hydroxymestanolone assay. The suppressed absorbance values obtained in the assay, relative to the negative control, are shown in the excretion curve in Fig. 6(a).

Alternatively, raw urine (1 mL) was treated by extraction on a Gilson Aspec XL4 liquid-handling system. The urine was adjusted to pH 5 and loaded onto an unconditioned reverse-phase SPE cartridge (30 mg, 1 mL, Absolut Nexus). The cartridge was washed with sodium hydroxide solution (0.1 M) and water and then eluted with methanol. The extract was evaporated to dryness at 70°C , under a stream of N_2 . Anhydrous 1 M HCl/methanol (0.5 mL) was added and the mixture were incubated at 60°C for 10 min. The solvolysate was then quenched with 2 mL of 0.25 M pH 8 phosphate buffer. Fifty microlitres of this quenched methanolysed extract (solvolysate) was analysed by the 16 β -hydroxymestanolone ELISA. The suppressed absorbances, relative to the negative control, are shown in the excretion curve in Fig. 6(b). The remainder of the quenched solvolysate was loaded onto an unconditioned mixed-mode (reverse-phase/cation exchange) SPE cartridge (30 mg, 1 mL, Oasis MCX) and washed with sodium hydroxide solution (0.1 M), then HCl solution (0.1 M) and then water. Neutral steroids were eluted with ethyl acetate (neutral eluate). After a methanol wash, basic steroids were eluted with diethylamine/ethyl acetate (2%, v/v). The basic eluate was dried at 70°C under a stream of N_2 . The residue was reconstituted in phosphate buffered saline (200 μL) and 50 μL was analysed using the 16 β -hydroxymestanolone assay. The suppressed absorbance values obtained are depicted in the excretion curve in Fig. 6(c).

(a)

(b)

(c)

Fig. 6. Excretion curves, determined using the 16 β -hydroxymestanolone ELISA plate, detecting stanazolol metabolites in equine urine, as shown by the absorbance suppression relative to the negative control. Following the intramuscular injection of 250 mg of stanazolol, the analyses were performed on urine which was: (a) hydrolysed with β -glucuronidase (1/100 dilution), (b) subjected to reverse-phase SPE, methanolysed and quenched and (c) subjected to mixed-mode SPE to give a basic eluate.

2.3.6.2. Analysis of mestanolone assay with equine urine following methandriol (27) administration. Raw urine and hydrolysed urine did not show significant suppression of absorbance in this assay. However, urine treated via reverse-phase SPE and subsequently methanolysed and quenched (as described for stanazolol) showed suppressed absorbances relative to the negative control (see Fig. 7(a)). Further treatment of the urine by mixed-mode SPE gave neutral eluates (as

(a)

(b)

Fig. 7. Excretion curves, determined using the mestanolone ELISA plate, detecting methandriol metabolites in equine urine, as shown by the absorbance suppression relative to the negative control. Following the intramuscular injection of 375 mg of methandriol dipropionate, the analyses were performed on urine which was (a) subjected to reverse-phase SPE, then methanolysed and quenched and (b) treated via mixed-mode SPE to give a neutral eluate.

for stanazolol), which gave suppressed absorbance measurements, relative to the negative control, in the excretion curve (see Fig. 7(b)).

2.3.6.3. Analysis of 16 β -hydroxymestanolone assay with equine urine following methandriol (27) administration. Methanolysed/quenched reverse-phase SPE treated urine and neutral eluates subsequently obtained after mixed-mode SPE (as described for stanazolol) gave suppressed absorbance measurements, as shown in Fig. 8.

2.3.7. GCMS analysis of equine urine following methandriol (27) administration

2.3.7.1. Sample preparation. Duplicate aliquots of urine (3 mL) were adjusted to pH 7 and spiked with 19-nor-5 α ,17 α -pregnane-3 β ,17 β -diol (20 ng mL⁻¹) as an internal standard. The samples were centrifuged (2000 $\times$ g, for 5 min) to sediment particulate matter, after which the supernatant fractions were loaded onto unconditioned Absolut Nexus solid-phase extraction cartridges (60 mg, 3 mL). The cartridges were rinsed with sodium hydroxide solution (0.1 M, 2 mL) and water (2 mL), and then dried briefly with air. The steroids

(a)

(b)

Fig. 8. Excretion curves, determined using a 16 β -hydroxymestanolone ELISA plate, detecting methandriol metabolites in equine urine, as shown by the absorbance suppression relative to the negative control. Following the intramuscular injection of 375 mg of methandriol dipropionate, the analyses were performed on urine which was (a) subjected to reverse-phase SPE, then methanolysed and quenched and (b) treated via mixed-mode SPE to give a neutral eluate.

were eluted with methanol (2 mL) and dried, by evaporation at 80 °C, under a stream of nitrogen. Steroidal sulfate and β -glucuronide conjugates were cleaved by reconstituting in anhydrous methanolic HCl (1 M, 0.5 mL) and incubating for 10 min at 60 °C. The reaction was quenched by the addition of sodium hydroxide solution (2 M, 4 mL) and extracted with diisopropyl ether (4 mL). The organic extracts were dried by evaporation at 80 °C, under a stream of nitrogen. The residues were reconstituted in acetonitrile (80 μ L) and pentafluoropropionic anhydride (20 μ L) and incubated for 15 min at 60 °C, and then dried, by evaporation at 80 °C, under a stream of nitrogen. The derivatised residues were reconstituted in ethyl acetate (50 μ L) for GCMS analysis.

2.3.7.2. GCMS analysis. Sample injections were made in split/splitless mode with an injector temperature of 250 °C. The column was initially held at 100 °C for 1 min, then ramped at 25–300 °C min⁻¹ and held for a further 1 min. Head pressure was programmed to maintain a constant flow rate of 0.5 mL min⁻¹ and the purge valve remained off for 1 min before opening to a split of 50:1. The MS transfer line

Fig. 9. Excretion curve, determined by GCMS analysis, for methandriol in equine urine following the intramuscular injection of 375 mg of methandriol dipropionate. The analysis was performed on urine subjected to reverse-phase SPE, methanolysis and quenching, followed by derivatisation with pentafluoropropionic anhydride.

was set at 280 °C and ionisation energy at 70 eV. Data were acquired in selected ion monitoring (SIM) mode, monitoring the ions m/z 417 for methandriol and m/z 405 for 19-nor-5 α ,17 α -pregnane-3 β ,17 β -diol (internal standard). The excretion curve obtained for methandriol (**27**) is shown in Fig. 9.

2.3.7.3. Quantitation. A duplicate calibration curve was constructed in blank equine urine over the range 0–40 ng mL⁻¹. The curve was linear over the stated range with a correlation coefficient (R^2) of 0.990. Duplicate quality control spikes at 20 ng mL⁻¹ returned concentration values within 5% of that expected. Further method validation was not performed.

3. Results and discussion

3.1. Synthesis

The aim of the synthetic studies was to diastereoselectively synthesise a representative 16,17-dihydroxy-17-alkyl anabolic steroid, and produce steroid-protein conjugates in order to raise antibodies that recognise the D-ring structure common to a number of steroid metabolites.

For this purpose, 16 β -hydroxymestanolone (**9**) was chosen as the representative steroid and was synthesised in six steps from commercially available epiandrosterone (**12**). As shown in Fig. 10, compound **13** was synthesised by published procedures [29–31]. Acetonide deprotection of **13** to yield **9** was achieved best using a Dowex 50W-X8 ion-exchange resin in methanol/water. Under these conditions, epimerisation of the C17 tertiary alcohol was minimised, such that a 14:1 ratio of **9** to **14** (17-*epi*-**9**) was obtained.

Thus, 16 β -hydroxymestanolone (**9**), together with commercially available mestanolone (**10**) and 17 α -methyltestosterone (17 β -hydroxy-17 α -methylandroster-4-en-3-one)

Fig. 10. Synthesis of 16β-hydroxymestanolone (9).

(11), were available for conjugation to HSA, to produce the antigenic material. While the primary interest was to generate antibodies that specifically recognised the 16β,17β-dihydroxy-17α-alkyl D-ring motif (see 9), antibodies were also raised against the parent steroidal 17β-hydroxy-17α-alkyl D-ring structure of 10 to enable a direct comparison of the selectivity of the antibodies formed. In addition, 17α-methyltestosterone (11) was conjugated to HSA to allow determination of the number of steroid residues attached per

protein by UV spectroscopic methods. Optimisation of this ratio is important for the generation of antibodies specific for the steroid.

In order to link the steroids to HSA at a distal position to the D-ring, a carboxymethyloxime tether to the A-ring via the 3-keto groups was chosen. Each of 16β-hydroxymestanolone (9), mestanolone (10) and 17α-methyltestosterone (11) was converted to the corresponding oximes 15–17 by reaction with carboxymethoxyamine hemihydrochloride (Fig. 11).

Fig. 11. Conjugation of steroids to HSA via two different peptide-coupling methods: direct carbodiimide activation or *N*-hydroxysuccinimide ester activation.

The carboxylic acid moiety then enabled the steroids to be linked via peptide-coupling techniques to the lysine residues of HSA to provide the antigenic material.

Two peptide-coupling techniques were employed to link the steroid to the protein, as shown in Fig. 11. Firstly, the carboxylic acid **15–17** was coupled directly to HSA using in situ carbodiimide activation (EDC/DCC) to afford conjugates **21–23**. Secondly, the activated *N*-hydroxysuccinimide ester method was utilised, as was first applied to certain steroids in their conjugation with BSA [35]. Hence, carboxylic acids **15–17** were converted to the activated esters **18–20** by treatment with *N*-hydroxysuccinimide in the presence of EDC. The activated esters **18–20**, upon treatment with HSA, then gave conjugates **24–26**. Low molecular weight impurities were removed from the conjugates by dialysis. In the case of **23** and **26**, further fractionation of the conjugates was achieved by adjusting the pH to 4.5, and separating the soluble from the insoluble material by centrifugation. As a result, **23a** and **26a** correspond to the fraction of the conjugates insoluble at pH 4.5, and **23b** and **26b** are the fractions soluble at this pH.

The relative efficiencies of the two coupling methods (carbodiimide versus *N*-hydroxysuccinimide ester activation) were assessed using conjugates **23** and **26**, which featured α,β -unsaturated oxime moieties and were, therefore, suitable for UV analyses. The α,β -unsaturated oxime of **17** was determined to have $\lambda_{\max}$ of 254 nm, and the extinction coefficient (ϵ) at this wavelength was determined to be $15,680 \text{ L mol}^{-1}$. The steroid/HSA molar ratios were then determined for **23** and **26** by UV spectral analyses at 254 nm in pH 7.5 buffer (0.1 M). The absorbances of the conjugates were compared with that of HSA at the same concentrations, and calculations were performed by the method of Erlanger et al. [36] using a molecular weight of HSA as $66,462 \text{ g mol}^{-1}$ and the extinction coefficient (ϵ) for **17**, which was assumed to remain the same when the steroids were conjugated to HSA. While this assumption is reported to give a relative error of $\sim 10\%$ due to the shift in the steroid **17** absorption maximum upon conjugation [38,39], it remains constant for conjugates of a given type, thus enabling comparisons of the same conjugates produced by different methods [40]. The results of these steroid-HSA molar ratio calculations are shown in Table 1 for **23a**, **23b** and **26a**. The pH 4.5 insoluble fraction **23a** gave a higher molar ratio of steroid to HSA than the corresponding pH 4.5 soluble fraction **23b**, consistent with the protonation of the remaining free HSA lysine residues aiding water solubility in the latter. The highest ratio of steroid/HSA of 28 was obtained for pH 4.5 insoluble fraction **26a**. The higher molar ratio obtained for conjugate **26** versus **23** revealed that conjugation of steroid to protein was much more effective using the *N*-hydroxysuccinimide ester activation (to give **26**). In fact, by this method of activation, the pH 4.5 soluble fraction **26b** contained very little soluble HSA conjugate, as indicated by the low absorbance of **26b** at $\lambda_{\max}$ of HSA of 208 nm. At pH 4.5, a negligible amount of conjugate **26** was water soluble, as surface lysine residues were not available for protonation. The

molar ratio of steroid/HSA was, therefore, not determined for **26b**. These observations were consistent with the high level of conjugation achieved via the *N*-hydroxysuccinimide ester activation versus the carbodiimide activation method, and the high molar ratio observed for **26a**. Hence, the *N*-hydroxysuccinimide ester activation maximises the number of steroid molecules attached to the protein surface. This is also consistent with the higher antibody titres (refer to Section 3.2) achieved for the conjugates **24** and **25** (derived via the *N*-hydroxysuccinimide ester method) versus those obtained for conjugates **21** and **22** (derived via the carbodiimide activation method), indicative of a higher steroid-HSA ratio for conjugates **24** and **25** versus **21** and **22**.

3.2. Antibody production and titre

For each conjugate **21**, **22**, **24** and **25**, four sheep were vaccinated and the sera collected. Antibody titres were then determined by an ELISA method. 16β -Hydroxymestanolone derivative **15** conjugated to ovalbumin was immobilised on plates and used in combination with antisera produced against conjugates **21** and **24**, and the corresponding antibody titres determined. As shown in Table 2, sera from sheep vaccinated with conjugate **24** gave higher titres than sera from sheep given conjugate **21**. Mestanolone derivative **16** immobilised on plates in the same way was used to determine antibody titres for antisera produced against conjugates **22** and **25** (refer to Table 2). The treatment with vaccine containing conjugate **25** gave higher antibody titres than vaccine containing **22**. Conjugates **24** and **25** were produced by *N*-hydroxysuccinimide coupling methods. The higher antibody titres observed using these conjugates also correlates with the higher steroid/protein molar ratio calculated for conjugate **26** versus that observed for **23** (Table 1, Sections 2.1.3 and 3.1). Hence, the *N*-hydroxysuccinimide coupling method gives a comparatively better conjugate for the production of antisera with high titres.

The sera from the two highest titre sheep per treatment were then collected and stored. The highest titre sera, raised in sheep against conjugates **24** and **25**, were used in all subsequent ELISA experiments. The 16β -hydroxymestanolone plate used in combination with antisera produced against conjugate **24**, constitutes the *16\beta*-hydroxymestanolone assay. The mestanolone plate used in combination with antisera against conjugate **25** forms the *mestanolone assay*.

3.3. ELISA assay and cross-reactivity studies

A number of anabolic steroids dissolved in assay buffer were tested by competition experiments using the 16β -hydroxymestanolone assay and the mestanolone assay, to assess cross-reactivities. The results for the 16β -hydroxymestanolone assay are shown in Table 3. High cross-reactivities (100–84%) were observed for all $16\beta,17\beta$ -dihydroxy- 17α -methyl steroids tested, therefore the antibody was highly specific for steroids **9** and **28–31**, as shown in Fig. 12.

Fig. 12. Anabolic steroids with high cross-reactivities in the 16 β -hydroxymestanolone assay.

Interestingly, one 16 α ,17 β -dihydroxy-17 α -methyl steroid, namely 16 α -hydroxyfurazabol (17 α -methyl-1',2',5'-oxadiazolo[4',3':2,3]-5 α -androstane-16 α ,17 β -diol) (**32**), also showed significant cross-reactivity (39.8%), whereas the other one tested, 17 α -methyl-5 α -androstane-3 β ,16 α ,17 β -triol exhibited low cross-reactivity (1.1%). Of 50 additional steroids tested, all showed <6% cross-reactivities. The antibodies raised against **24** showed low affinity for steroids that lacked a 16-hydroxy substituent, and/or those that lacked a 17 α -alkyl substituent. The 16,17-dihydroxy-17 α -ethyl steroids tested also gave low cross-reactivities (19-nor-5 α ,17 α -pregnane-3 β ,16 β ,17 β -triol (5.5%) and 19-nor-5 α ,17 α -pregnane-3 β ,16 α ,17 β -triol (<1%)). The antibodies raised against **24** selectively recognise the 16 β ,17 β -dihydroxy-17 α -methyl D-ring structure common to many anabolic steroid metabolites, and so the 16 β -hydroxymestanolone assay shows promise for the detection of this class of steroid metabolite.

The cross-reactivity results for the mestanolone assay, showing the specificity of the antibodies raised against **25**, are shown in Table 4. These antibodies showed significant cross-reactivities for most 17 β -hydroxy-17 α -methyl steroids, although for this antibody, changes in the A, B and C rings also influenced the cross-reactivities. For example, while 17 α -methyltestosterone gave a cross-reactivity of 100%, a minor change in the A-ring in methandrostenolone (17 β -hydroxy-17 α -methylandroster-1,4-dien-3-one) or in the B-ring in bolasterone (17 β -hydroxy-7 α ,17 α -dimethylandroster-4-en-3-one) resulted in cross-reactivities of 28.1 and 25.2%, respectively. 17 β -Hydroxy-17 α -ethynyl steroids also exhibited significant cross-reactivities. In general, for these antibodies, lower cross-reactivities were observed for steroids with a 16-hydroxy substituent and for steroids that lacked a 17 α -alkyl substituent. The cross-reactivities observed for 17 β -hydroxy-17 α -ethyl steroids were mixed. Hence, the antibodies raised against **25** recognise the 17 β -hydroxy-

17 α -methyl D-ring structure common to many parent anabolic steroids, but were not as selective as the 16 β -hydroxymestanolone assay. As a consequence, some endogenous steroids, e.g. testosterone, which may be excreted in high concentrations, may be problematic in the mestanolone assay despite relatively low cross-reactivities (e.g. testosterone (5.6%)).

3.3.1. Validity of the cross-reactivity studies

As shown in Figs. 4 and 5, the B/B_0 absorbances were determined by the ELISA assays for a number of the cross-reactive anabolic steroid standards, together with the original steroid, 16 β -hydroxymestanolone (for the 16 β -hydroxymestanolone plate) or mestanolone (for the mestanolone plate). Plots of percentage absorbance versus concentration show that those steroid standards that had significant cross-reactivity ran parallel to the original compounds. By contrast, those steroid standards that were less cross-reactive did not follow this trend. These results indicated that the cross-reactivity comparisons were valid for these assays.

3.4. Application to administration urine samples

Stanozolol (**4**) and methandriol (**27**) (refer to Fig. 13) were administered to horses and the urine samples were then analysed by the 16 β -hydroxymestanolone assay to assess detection of the 16 β -hydroxy metabolites. Urine samples post methandriol (**27**) administration were analysed by the mestanolone assay, GCMS and the 16 β -hydroxymestanolone assay.

Because of low urinary excretion and rapid metabolism, stanozolol (**4**) is difficult to detect in urine. Instead, methods of detecting metabolites of **4** have been sought. The metabolites are mostly excreted as glucuronide or sulfate conjugates. Hence, enzymatic hydrolysis and extraction procedures pre-

Fig. 13. Anabolic steroids administered to horses for analysis of equine urine via the 16 β -hydroxymestanolone and mestanolone assays.

cede LCMS detection techniques with derivatisations also required for GCMS detection methods [28].

Since 16 β -hydroxystanozolol showed 100% cross-reactivity to the 16 β -hydroxymestanolone antigen, equine urine post-stanozolol (**4**) administration was analysed by the 16 β -hydroxymestanolone assay to screen for the presence of 16 β -hydroxystanozolol. Analysis of raw urine produced negative results. However, this is consistent with existing methods of analysis, which require hydrolysis and extraction procedures to release stanozolol metabolites present as glucuronide or sulfate conjugates [28].

Therefore, the raw urine was hydrolysed overnight with a 1/100 dilution of β -glucuronidase and then assayed. The absorbances obtained in the assays during the days post-administration are shown in Fig. 6(a). A decrease in absorbance, relative to the negative control (day 0), is indicative of competition between a substance in the urine sample and the parent antigen, for binding to the antibody. Similar suppressed absorbances were observed with different batches of β -glucuronidase hydrolysed raw urine and different batches of ELISA plates, and showed the “saw tooth” excretion pattern typically observed for long-acting anabolic steroid depot preparations [20,41–44]. Hence, cross-reacting stanozolol metabolites were detected in the assay.

The raw urine was then subjected to reverse-phase SPE, methanolysed and quenched with pH 8 buffer and analysed again in the 16 β -hydroxymestanolone assay. A further extraction of the residual quenched solvolysate (by mixed-mode reverse-phase/cation exchange SPE) was carried out and the basic extract also analysed in the 16 β -hydroxymestanolone assay. The absorbances obtained in the assays during the days post-administration are shown in Figs. 6(b and c). Again, decreased absorbances are observed relative to the negative control, with the excretion profile matching that observed by the standard LCMS method [20]. Since unmetabolised stanozolol (**4**) gives negligible cross-reactivity (refer to Table 3) and dihydroxylated stanozolol metabolites were not previously detected in equine urine samples [17,20], a comparison with the standard LCMS procedure [20] indicates the detections (particularly as in Fig. 6(c)), were primarily due to 16 β -hydroxystanozolol.

Methandriol (**27**) was administered to a horse and its urine was assessed via the mestanolone assay. Urine was analysed raw and following β -glucuronidase hydrolysis via the mestanolone assay, but negative results were obtained. Again,

metabolites are present significantly as glucuronide and sulfate conjugates, although the negative result following the hydrolysis step is consistent with interference by endogenous materials. Hence, the raw urine was extracted (SPE), methanolysed and quenched (as per stanozolol administered urine). Further extraction (SPE) of the quenched solvolysate was carried out, and both the quenched solvolysate and the neutral extract from subsequent SPE were analysed via the mestanolone assay. Suppressed absorbance readings were obtained up to 35 days post-administration for both extracts (Fig. 7). Hence, methandriol metabolites, presumably featuring a 17 β -hydroxy-17 α -methyl substituted D-ring, were detected by the mestanolone assay up to 35 days later. Analysis of the quenched solvolysate by GCMS revealed that methandriol (**27**) constituted the major component, and its excretion curve is shown in Fig. 9. The typical “saw tooth” pattern [20,41–44] observed correlates well with the pattern observed by the mestanolone assay.

In humans, methandriol (**27**), in contrast to stanozolol (**4**), undergoes extensive metabolism in the A and B rings, including double bond isomerisation, oxidation and reductions, with 17 α -methyltestosterone thought to be intermediary [24]. As shown in Table 4, methyltestosterone and 17 α -methyl-5 α -androstane-3 β ,17 β -diol, both potential metabolites of methandriol, both showed 100% cross-reactivity in the mestanolone assay. Methandriol itself also showed high cross-reactivity of 78.7%. Hence, the assay would be expected to detect these metabolites. Studies on the metabolism of 17 α -methyltestosterone in the horse have identified metabolites with and without further hydroxylation of the D-ring [16,18,19], but further D-ring hydroxylated metabolites would not be detected in this assay, as the cross-reactivities for such compounds were low (refer to Section 3.3). Only trace amounts of methyltestosterone and methylandrostanediols were detected in the GCMS analysis, and hence it is likely that the excretion of methandriol (**27**) accounts for the ELISA result.

In order to detect methandriol metabolites resulting from hydroxylation of the D-ring at position 16, the quenched solvolysate and neutral extract from subsequent SPE were also analysed by the 16 β -hydroxymestanolone assay, as shown in Fig. 8. As indicated by the suppressed absorbances, a metabolite or metabolites that cross-reacted with the plate were detected through to day 42. Further work will ascertain whether the presence of 16 β -hydroxylated metabolites

of methandriol in the urine account for this suppression of absorbance.

4. Conclusion

The ELISA methods developed are an effective screening tool for routine detection of new and known anabolic steroids and their metabolites in equine urine. In particular, the 16 β -hydroxymestanolone assay specifically detects a common D-ring metabolite structure of 17 α -methyl steroids. The mestanolone assay detects the corresponding parent steroids. Further assays will be developed involving antibodies raised against the 16 α -hydroxymestanolone D-ring structure and also against alternate 17-alkyl steroids and metabolites (e.g. ethyl or ethynyl) to broaden the scope of the screen, with potential to detect new designer steroids and metabolites, such as tetrahydrogestrinone.

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