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THE METABOLISM OF SYNTHETIC ANABOLIC-ANDROGENIC STEROIDS IN THE GREYHOUND: BOLDENONE UNDECYLENATE AND STANOZOLOL

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ABSTRACT

In mid-2008, Greyhounds Australasia, the main administrative body for greyhound racing in Australia and New Zealand, introduced new rules regulating the use of anabolic-androgenic steroids in racing greyhounds. To assist with the development of methodology to effectively police these rules, the Australian Racing Forensic Laboratory and the Research School of Chemistry at the Australian National University have entered into a 3 year collaborative research agreement with a view to identifying suitable targets for MS-based doping analysis. Metabolites of selected synthetic anabolic-androgenic steroids are investigated in post administration greyhound urine samples using both GC/MS and LC/MS techniques, and the structures of significant phase I metabolites are confirmed by comparison with authentic reference standards. Where such standards are not commercially available, efforts are made to synthesise and properly characterise them. Phase II metabolic studies are also undertaken. As a result, comprehensive theories of the canine metabolism of steroids including boldenone, ethylestrenol, mesterolone, methandriol, nandrolone, norethandrolone and stanozolol are being developed.

INTRODUCTION

Anabolic-androgenic steroid testing in greyhounds is a relatively underdeveloped science. Despite several decades of increasingly sophisticated steroid testing in the fields of human and equine athletics, only a handful of publications concerning the canine disposition of these substances has emerged to date. The available information, however, suggests that canine steroid metabolism is qualitatively different to either human or equine steroid metabolism, and, consequently, deserves to be treated in its own right.

Since 1st July 2008, greyhound racing in Australia has operated under strict new steroid rules. Under these rules, all anabolic-androgenic steroids are considered prohibited substances on race day with the exception of ethylestrenol, which is permitted in bitches for the purpose of oestrus control. To facilitate effective policing of the new prohibition, the Australian Racing Forensic Laboratory has entered into a collaboration with a leading university chemistry department to perform fresh research into canine anabolic-androgenic steroid metabolism. At present, administration trials for boldenone undecylenate, ethylestrenol, mesterolone and stanozolol have been analysed, and syntheses of the principal phase I metabolites of these steroids are in progress. Results for boldenone undecylenate and stanozolol are presented here. Further administration trials involving additional steroids are planned.

MATERIALS AND METHODS

Chemicals and consumables

Androsta-1,4-diene-3,17-dione, boldenone, 3α-hydroxy-5α-androstan-17-one, 3α-hydroxy-5β-androstan-17-one, 3β-hydroxy-5α-androstan-17-one, methandriol, stanozolol and *E. coli* β-glucuronidase (Type IX-A; P/N G7396) were purchased from Sigma (New South Wales, Australia). 5α-Androst-1-ene-3,17-dione, 3β-hydroxy-5β-androstan-17-one and 17β-hydroxy-

5α-androst-1-en-3-one were purchased from Steraloids (Rhode Island, USA). 17-epiboldenone and 17β-hydroxy-5β-androst-1-en-3-one were purchased from the National Measurement Institute (New South Wales, Australia). 3'-hydroxystanozolol, 4α-hydroxystanozolol and 4β-hydroxystanozolol were purchased from Cerilliant (Texas, USA). 16β-hydroxystanozolol was synthesised under contract by BDG Synthesis (New Zealand). Oasis WAX solid phase extraction cartridges were purchased from Waters (New South Wales, Australia). Anhydrous methanolic hydrogen chloride (1 M) was prepared according to the method of Tang and Crone (1989).

Synthesis of reference materials

The following reference materials have thus far been produced on a small scale only. Full synthetic details will be published progressively as the syntheses are scaled up and the products rigorously characterised.

5β-androst-1-ene-3,17-dione: 5β-androst-1-ene-3,17-dione was prepared by the oxidation (pyridinium chlorochromate) of 17β-hydroxy-5β-androst-1-en-3-one.

5α-androst-1-ene-3α,17β-diol and 5α-androst-1-ene-3β,17β-diol: These compounds were prepared by the reduction (lithium aluminium hydride) of 17β-hydroxy-5α-androst-1-en-3-one. GC/MS analysis revealed the existence of 2 peaks in 10.1:1 ratio which were assigned as the 3β- and 3α-alcohols respectively in accordance with literature precedent (Bergmann *et al.* 1954).

5β-androst-1-ene-3α,17β-diol and 5β-androst-1-ene-3β,17β-diol: These compounds were prepared from 17β-hydroxy-5β-androst-1-en-3-one in an analogous manner to the 5α-isomers described above. Two GC/MS peaks in 6.5:1 ratio were assigned as the 3α- and 3β-alcohols respectively in accordance with literature precedent (Schänzer and Donike 1992).

6α-hydroxystanozolol: 6α-hydroxystanozolol was prepared from methandriol by double bond hydration (borane/hydrogen peroxide), selective oxidation of the 3-hydroxyl (*N*-bromosuccinimide), hydroxymethylenation at C2 (ethyl formate) and condensation with hydrazine.

6β-hydroxystanozolol: 6β-hydroxystanozolol was prepared from 6α-hydroxystanozolol by oxidation (chromium [VI] oxide) and reduction (sodium borohydride).

Administration trials

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

Boldenone undecylenate: An oily solution of boldenone undecylenate (Boldebal-H, Ilium, New South Wales, Australia; 1.1 mL = 55 mg steroid) was administered by intramuscular injection to one female greyhound (9 years, 27 kg). Urine samples were collected pre-administration, at 6 h post administration, then at 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 21 and 29 days post administration.

Stanozolol: An aqueous suspension of stanozolol (Stanabolic, Ilium, New South Wales, Australia; 1.4 mL = 70 mg steroid) was administered by intramuscular injection to one male greyhound (5 years, 35 kg). Urine samples were collected pre-administration, at 6 h post administration, then at 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 21 and 29 days post administration.

Sample preparation

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

Clean-up: unconjugated fraction: Dried eluates were reconstituted in methanol (0.5 mL). Sodium hydroxide solution (2 M, 4 mL) was added and the mixture was extracted with diisopropyl ether (4 mL). Stanozolol residues were further purified by extraction into sulphuric acid (0.35 M, 2 mL)

followed by basification with sodium hydroxide solution (2 M, 2 mL) and back-extraction into diisopropyl ether (4 mL). The final ether extract in each case was dried by evaporation at 80°C under nitrogen.

Hydrolysis and clean-up: glucuronide fraction: Dried eluates were reconstituted in sodium citrate buffer solution (pH 6, 0.1 M, 0.5 mL). An aqueous solution of *E. coli* β-glucuronidase (2 mg/mL, 50 μL) was added, and the mixture was incubated overnight at 37°C. Sodium hydroxide solution (2 M, 4 mL) was then added, and the mixture was extracted with diisopropyl ether (4 mL). Stanozolol residues were further purified by extraction into acid followed by basification and back-extraction as described above. The final ether extract in each case was dried by evaporation at 80°C under nitrogen.

Solvolysis and clean-up: sulphate fraction: Dried eluates were reconstituted in anhydrous methanolic hydrogen chloride (1 M, 0.5 mL) and incubated for 15 min at 60°C. Sodium hydroxide solution (2 M, 4 mL) was then added, and the mixture was extracted with diisopropyl ether (4 mL). Stanozolol residues were further purified by extraction into acid followed by basification and back-extraction as described above. The final ether extract in each case was dried by evaporation at 80°C under nitrogen.

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

GC/MS analysis: boldenone undecylenate

Dried residues were reconstituted in dodecane (50 μL) for GC/MS analysis. Data were acquired using a Waters-Micromass (New South Wales, Australia) Quattro triple quadrupole MS connected to an Agilent (New South Wales, Australia) 6890 Series GC. The column was a SGE (Victoria, Australia) BPX-5 (12 m x 0.25 mm id, 0.25 μm film thickness) with helium as carrier gas. Sample injections (2 μL) were made in splitless mode with an injector temperature of 250°C. The column was initially held at 100°C for 1 min, then ramped at 20°C/min to 300°C and held for a further 5 min. Head pressure was programmed to maintain a constant flow rate of 1 mL/min, and the purge valve was opened at 2 min to a split of 30:1. The MS was operated in electron ionisation (EI) mode with a transfer line temperature of 250°C, source temperature of 200°C and ionisation energy of 70 eV. Full scan data were collected over the range m/z 50–700 with a scan time of 250 ms. Selected ion monitoring (SIM) was performed with dwell times of 20 ms using a range of targets corresponding to the theoretical $M^{\cdot+}$ and $[M-57]^+$ signals of proposed metabolites incorporating common metabolic modifications such as oxidations, reductions or hydroxylations. Full scan MS/MS data were collected over the range m/z 50–700 with a scan time of 250 ms using argon as collision gas and activation energies optimised for individual compounds.

LC/MS analysis: stanozolol

Dried residues were reconstituted in methanol (50 μL) and ammonium acetate solution (50 mM, 50 μL) for LC/MS analysis. Data were acquired using a Thermo (New South Wales, Australia) LCQ Deca XP ion trap MS equipped with a Surveyor LC system. The column was a Waters (New South Wales, Australia) Xterra C18 (150 mm x 2.1 mm id, 3.5 μm particle size) protected by a Phenomenex (New South Wales, Australia) Security-Guard C18 (2 mm x 4 mm id) guard column. Sample injections (10 μL) were made into a mobile phase comprising 95% formic acid (25 mM) and 5% acetonitrile. After holding for 0.5 min, the proportion of acetonitrile was increased to 95% in a linear gradient over 4.5 min. This composition was held for a further 4 min, then was returned to starting conditions and equilibrated for 2 min prior to the next injection. The flow rate was maintained at 200 μL/min throughout. The MS was operated in positive ion electrospray ionisation mode with source conditions optimised for stanozolol. Precursor ions corresponding to the proton adducts of stanozolol (m/z 329), monohydroxystanozolols (m/z 345) and dihydroxystanozolols (m/z 361) were isolated and fragmented with individually optimised parameters. Full scan MS/MS data were collected over the range m/z 100–400 in each case.

RESULTS AND DISCUSSION

Analytical procedures

Past investigations of phase II anabolic steroid metabolism in various species have typically

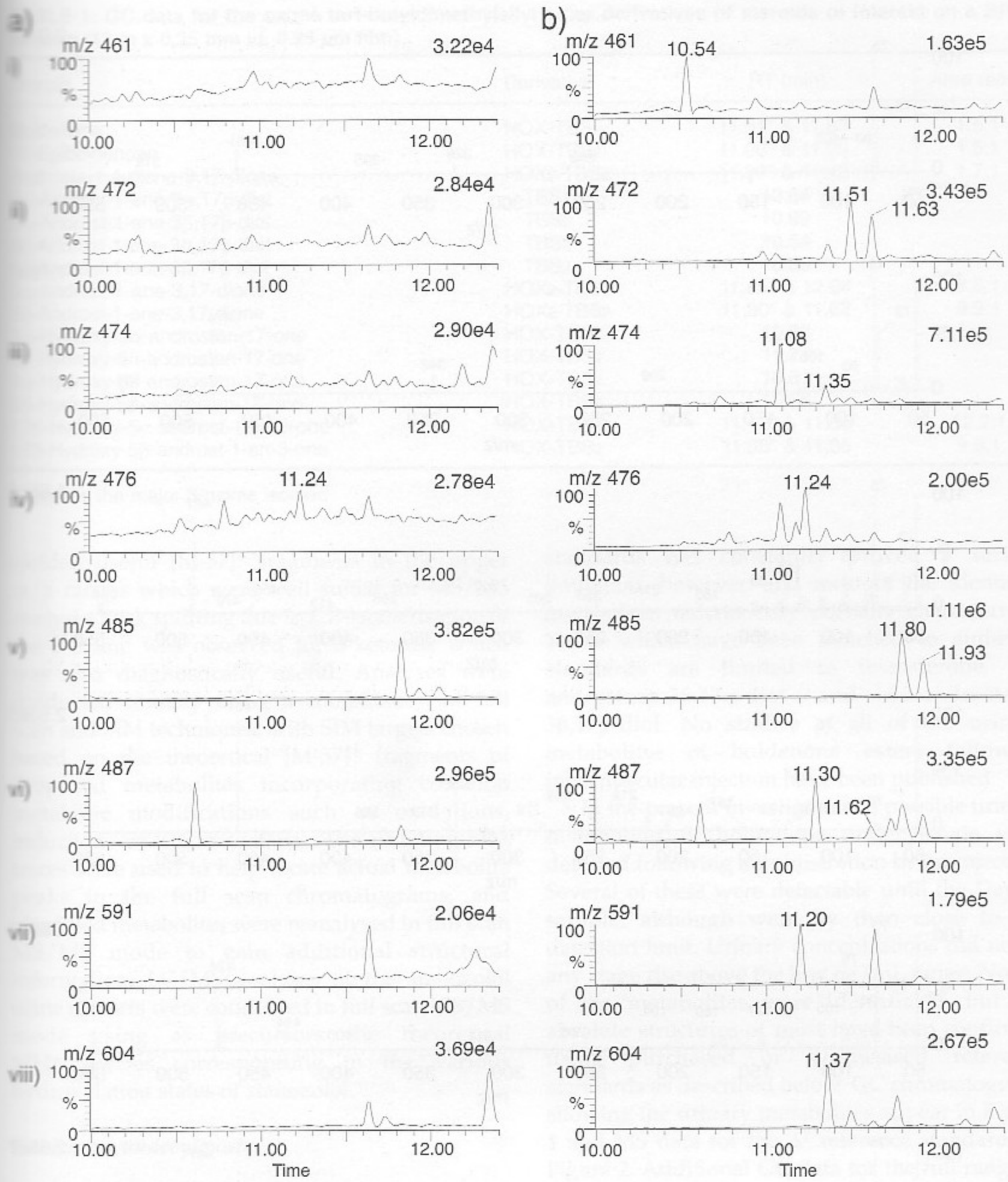

Fig 1: Extracted ion chromatograms from: a) control; and b) 48 h post administration urine samples showing peaks corresponding to the oxime and/or tert-butyldimethylsilyl ether derivatives of: i) 5β-androst-1-ene-3α,17β-diol; ii) boldenone; iii) 17β-hydroxy-5β-androst-1-en-3-one; iv) 3β-hydroxy-5β-androstan-17-one; v) androsta-1,4-diene-3,17-dione; vi) 5β-androst-1-ene-3,17-dione; vii) an unidentified androstenetriol; and viii) an unidentified dihydroxyandrostenone.

relied either on gel filtration techniques or on serial extractions punctuated by conjugate-specific hydrolysis steps to generate separate unconjugated, β-glucuronide conjugated and sulphate conjugated steroid fractions. These approaches are both labour intensive and time consuming, however, and so a third technique was developed for the present application. This involved the fractionation of unconjugated, β-glucuronide conjugated and sulphate conjugated steroids in a single solid phase extraction step using a mixed mode weak anion exchange sorbent (Oasis WAX) to take advantage of the differing pKa values of the acidic steroid conjugates. Steroids in aqueous media bind to the sorbent initially via a reversed phase mechanism, after which careful pH control allows selective elution of the various conjugate groups. Unconjugated steroids are eluted at pH 7.5, leaving the ionised steroid conjugates bound to the protonated anion

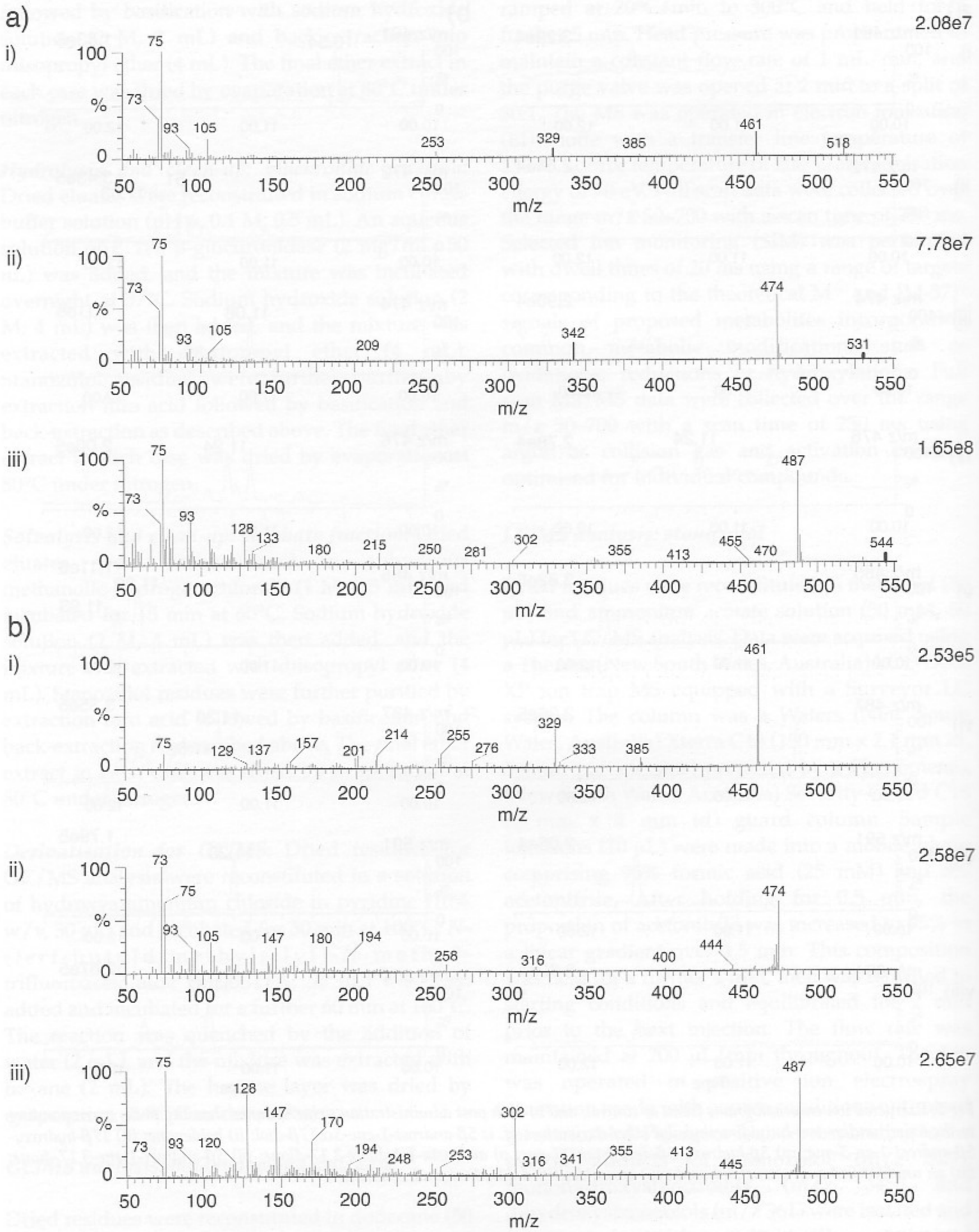

Fig 2: a) Full scan MS; and b) full scan MS/MS spectra for the oxime and/or tert-butyldimethylsilyl ether derivatives of: i) 5β-androst-1-ene-3α,17β-diol: ii) 17β-hydroxy-5β-androst-1-en-3-one; and iii) 5β-androst-1-ene-3,17-dione.

exchanger. A subsequent acidic elution sees protonation and release of the weakly acidic β-glucuronic acid conjugates, and a final basic elution deprotonates the ion exchanger and releases the strongly acidic sulphuric acid conjugates. The 3 fractions so obtained are then hydrolysed as necessary and further cleaned up by solvent extraction. This method proved to be quick and robust, and was applied in all of the studies described here.

Extraction residues bound for GC/MS analysis were then derivatised by oximination followed by *tert*-butyldimethylsilylation as described by Teale and Houghton (1991). These derivatives typically

TABLE 1: GC data for the oxime tert-butyldimethylsilyl ether derivatives of steroids of interest on a BPX-5 column (12 m x 0.25 mm id, 0.25 μm film)

Steroid	Derivative	RT (min)	Area ratio
Boldenone	HOX-TBS2	11.51* & 11.63	1.5:1
17-Epiboldenone	HOX-TBS2	11.00* & 11.09	1.5:1
Androsta-1,4-diene-3,17-dione	HOX2-TBS2	11.80* & 11.93	1.7:1
5α-Androst-1-ene-3α,17β-diol	TBS2	10.64	
5α-Androst-1-ene-3β,17β-diol	TBS2	10.99	
5β-Androst-1-ene-3α,17β-diol	TBS2	10.54	
5β-Androst-1-ene-3β,17β-diol	TBS2	10.68	
5α-Androst-1-ene-3,17-dione	HOX2-TBS2	11.85* & 12.04	8.6:1
5β-Androst-1-ene-3,17-dione	HOX2-TBS2	11.30* & 11.62	9.9:1
3α-Hydroxy-5α-androstan-17-one	HOX-TBS2	10.82	
3β-Hydroxy-5α-androstan-17-one	HOX-TBS2	11.24	
3α-Hydroxy-5β-androstan-17-one	HOX-TBS2	10.81	
3β-Hydroxy-5β-androstan-17-one	HOX-TBS2	10.66	
17β-Hydroxy-5α-androst-1-en-3-one	HOX-TBS2	11.42* & 11.58	12.2:1
17β-Hydroxy-5β-androst-1-en-3-one	HOX-TBS2	11.08* & 11.35	9.6:1

*Denotes the major 3-oxime isomer.

yielded useful $[M-57]^+$ fragments in the upper m/z ranges which were well suited for MS/MS analysis. Peak splitting due to *E,Z*-isomerisation of the 3-oxime was observed for 3-ketones, which was also diagnostically useful. Analyses were conducted initially using a combination of full scan and SIM techniques, with SIM targets chosen based on the theoretical $[M-57]^+$ fragments of proposed metabolites incorporating common metabolic modifications such as oxidations, reductions or hydroxylations. The resulting SIM traces were used to help locate actual metabolite peaks in the full scan chromatograms, and identified metabolites were reanalysed in full scan MS/MS mode to gain additional structural information. LC/MS analyses of the stanozolol urine extracts were conducted in full scan MS/MS mode using as precursors the theoretical MH^+ species corresponding to the various hydroxylation states of stanozolol.

Boldenone undecylenate

Boldenone and its esters are not registered for small animal use in Australia, but the undecylenate is available in some large animal preparations with potential for off label use in greyhounds. Three previous studies of canine boldenone metabolism have been performed, but have provided only a limited insight into the subject. *In vitro* incubation of boldenone with beagle liver microsomes (Williams *et al.* 2000a) plus oral (Williams *et al.* 2000b,c) and iv (Brockwell *et al.* 1992) boldenone administration trials in greyhounds have indicated extensive metabolism involving complex combinations of oxidative and reductive processes as well as additional oxygenation. The absence of suitable reference standards has constantly proved a serious limitation, however, and most of the identified metabolites remain only partially characterised. Those which have been matched to authentic standards are limited to testosterone, 5α-androstane-3β,17α-diol and 5α-androstane-3β,17β-diol. No studies at all of the urinary metabolites of boldenone esters following intramuscular injection have been published.

In the present investigation, 8 possible urinary metabolites of boldenone undecylenate were detected following administration by im injection. Several of these were detectable until the Day 29 sample, although were by then close to the detection limit. Urinary concentrations did not at any stage rise above the low ng/mL range. Not all of the metabolites were identifiable, but the absolute structures of most have been confirmed using purchased or synthesised reference standards as described below. GC chromatograms showing the urinary metabolites appear in Figure 1 and MS data for the Δ^1 reference standards in Figure 2. Additional GC data for the full range of steroids examined appear in Table 1.

i) A pair of peaks appearing mainly in the β-glucuronide fraction and to a lesser extent in the unconjugated fraction were identified as boldenone after comparison with commercial standards of boldenone and 17-epiboldenone.

ii) A pair of peaks appearing in the unconjugated fraction until Day 14 were identified as androsta-1,4-diene-3,17-dione after comparison with a commercial standard.

iii) A pair of peaks appearing in the unconjugated fraction until Day 14 were identified as 5β-androst-1-ene-3,17-dione after comparison with a synthesised standard of this compound

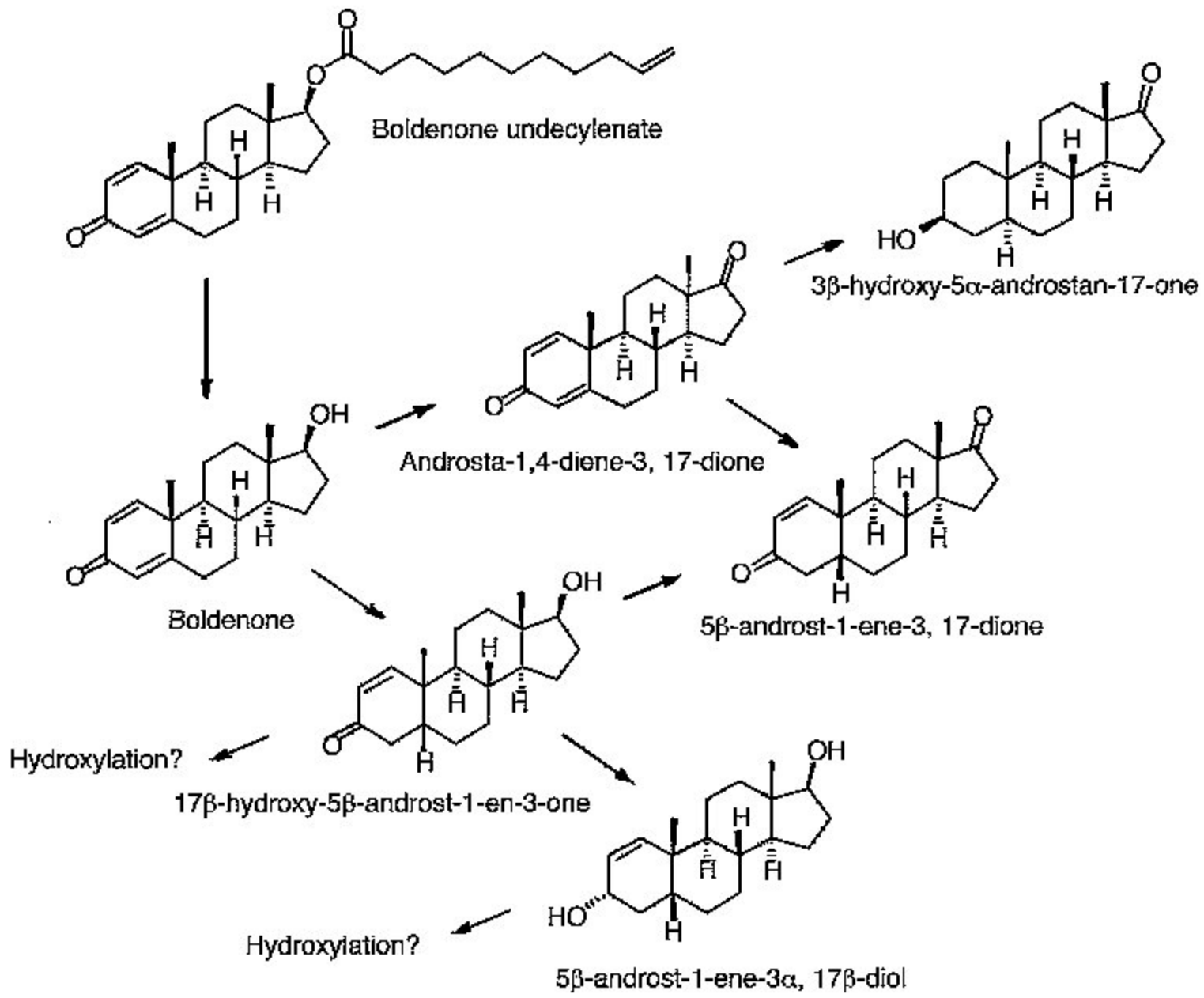

Fig 3: Proposed phase I metabolism for boldenone undecylenate in the greyhound following administration by intramuscular injection.

and a commercial standard of its 5-epimer, 5α-androst-1-ene-3,17-dione.

iv) A peak appearing in the β-glucuronide fraction was identified as 3β-hydroxy-5α-androstan-17-one (epiandrosterone) after comparison with commercial standards of this compound and its 3 C3/C5-stereoisomers. There did appear to be a trace of epiandrosterone in the control sample and it is likely that a basal concentration of endogenous origin was present as reported by Biddle *et al.* (2005). Furthermore, after an initial marked rise in urinary concentration starting with the earliest post administration samples, the levels did not tail off in the same manner as the other steroids described, but instead remained clearly elevated until the final sample collected on Day 29. Whether this phenomenon was due to the direct metabolism of boldenone to epiandrosterone or rather to a stimulation of endogenous epiandrosterone production promoted by the boldenone administration has not yet been established.

v) A peak with a similar phase II distribution to boldenone was identified as 17β-hydroxy-5β-androst-1-en-3-one after comparison with commercial standards of this compound and its 5-epimer, 17β-hydroxy-5α-androst-1-en-3-one. No 17α-hydroxy stereoisomers were available for comparison, and thus the possibility of 17-epimerisation cannot be completely discounted.

vi) A peak appearing in the β-glucuronide fraction until Day 21 was identified as 5β-androst-1-ene-3α,17β-diol after comparison with synthesised standards of this compound and its 3 C3/C5-stereoisomers. No 17α-hydroxy stereoisomers were available for comparison, thus once again leaving open the possibility of 17-epimerisation.

vii) In addition to the above, 2 observed peaks remain as yet unidentified. From their mass spectra, these are thought to correspond to a dihydroxyandrostenone ($[M-57]^+$ = m/z 604) and an androstenetriol ($[M-57]^+$ = m/z 591). Investigations are continuing.

Based on the above, a number of phase I metabolic processes for boldenone following im injection of the undecylenate ester may be proposed (Fig 3). Reduction of the A-ring with 5α,3β-

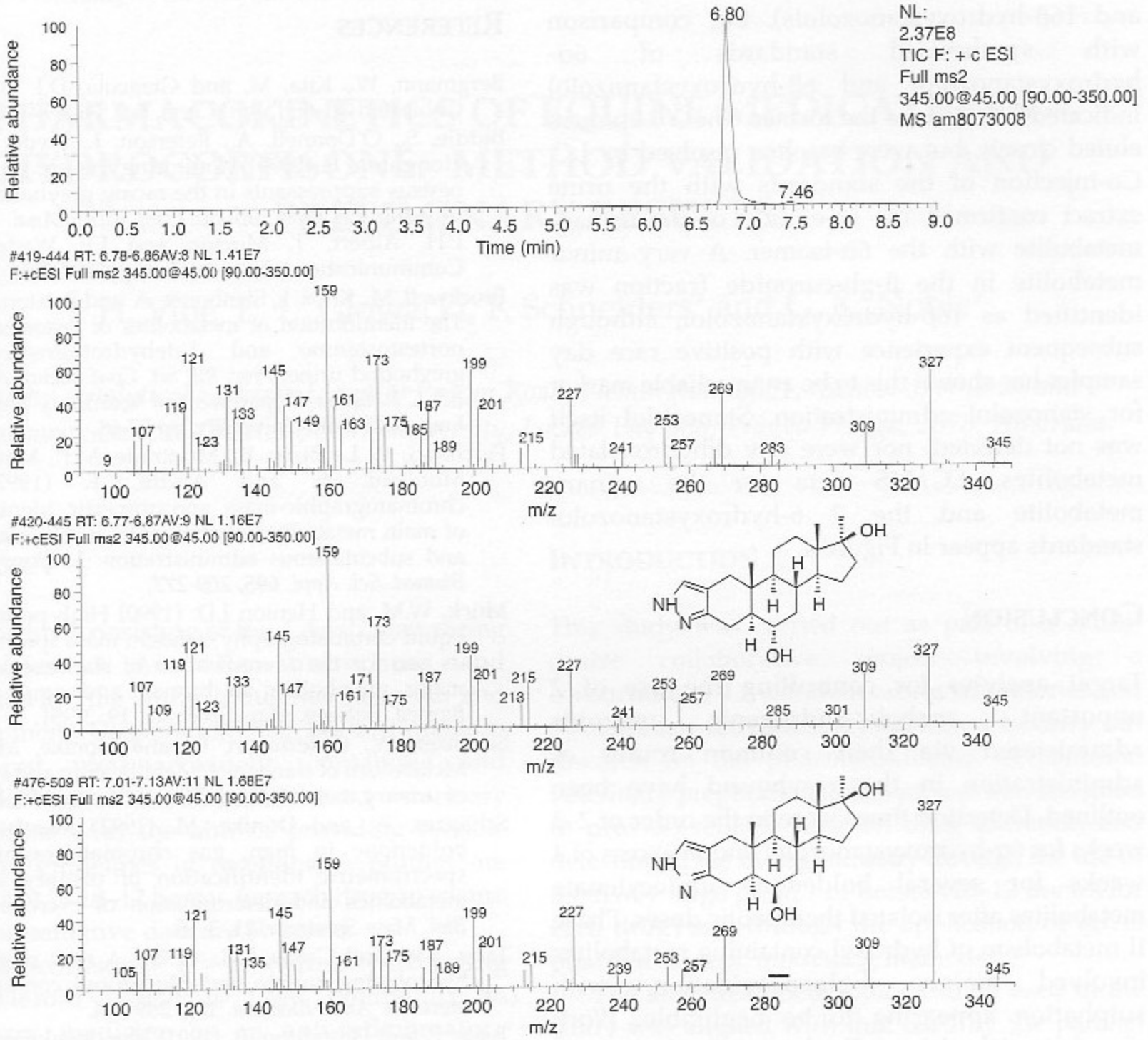

Fig 4: i) LC chromatogram; and ii) full scan ion trap product ion spectrum for the major canine urinary stanozolol metabolite together with corresponding spectra for iii) 6α-hydroxystanozolol and iv) 6β-hydroxystanozolol. The precursor ion in each case was m/z 345.

stereochemistry appears to be rapid and complete, as evidenced by the absence of any intermediate reduction products in the urine. An initial reduction of the Δ^4-unsaturation with 5β-stereochemistry on the other hand appears to inhibit subsequent reductive steps, leading to the presence of Δ^1-3-keto and Δ^1-3α-hydroxy metabolites in the urine. No saturated 5β-androstanes were detected, suggesting the Δ^1-5β-configuration to be immune to further reduction. Oxidation to the 17-ketone also occurs, although appears to be more important in conjunction with the 5α-reduction pathway than the 5β. No evidence of subsequent reduction to the 17α-hydroxyl was observed, although it must be noted that standards corresponding to this configuration were generally unavailable for comparison. Hydroxylation of the steroid nucleus as described in the earlier boldenone studies was apparent, although further details can not be elaborated at this stage. Phase II metabolism in general was found to involve mainly β-glucuronidation, with some metabolites being wholly or partially unconjugated. No sulphate conjugated metabolites were detected.

Stanozolol

Stanozolol is known to undergo complex metabolism in man (Schänzer *et al.* 1990), cattle (Ferchaud *et al.* 1997) and horses (Mück and Henion 1990), with hydroxylation at C3', C4 and C16 being the most important metabolic pathways. In the greyhound, a major hydroxylated metabolite was detected in the β-glucuronide fraction until the Day 14 sample following the im injection of an aqueous suspension of stanozolol. No match was obtained with any of the commercially available monohydroxystanozolol standards (3'-, 4α-, 4β-

and 16β-hydroxystanozolols), but comparison with synthesised standards of 6α-hydroxystanozolol and 6β-hydroxystanozolol indicated a match for the former. The 2 6-epimers eluted closely, but were baseline resolved by LC. Co-injection of the standards with the urine extract confirmed the co-elution of the urinary metabolite with the 6α-isomer. A very minor metabolite in the β-glucuronide fraction was identified as 16β-hydroxystanozolol, although subsequent experience with positive race day samples has shown this to be an unreliable marker for stanozolol administration. Stanozolol itself was not detected, nor were any dihydroxylated metabolites. LC/MS data for the urinary metabolite and the 2 6-hydroxystanozolol standards appear in Figure 4.

Conclusion

Target analytes for controlling the use of 2 important anabolic-androgenic steroids administered via their common routes of administration in the greyhound have been outlined. Detection times were in the order of 2–3 weeks for 6α-hydroxystanozolol and in excess of 4 weeks for several boldenone undecylenate metabolites after isolated therapeutic doses. Phase II metabolism of hydroxyl containing metabolites involved mainly β-glucuronidation, with sulphation appearing to be negligible. Work aimed at synthesising fully characterised reference standards for all the major phase I metabolites is ongoing.

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