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Detection of stanozolol and its metabolites in equine urine by liquid chromatography–electrospray ionization ion trap mass spectrometry

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Abstract

The equine phase I and phase II metabolism of the synthetic anabolic steroid stanozolol was investigated following its administration by intramuscular injection to a thoroughbred gelding. The major phase I biotransformations were hydroxylation at C16 and one other site, while phase II metabolism in the form of sulfate and β-glucuronide conjugation was extensive. An analytical procedure was developed for the detection of stanozolol and its metabolites in equine urine using solid phase extraction, acid solvolysis of phase II conjugates and analysis by positive ion electrospray ionization ion trap LC–MS.

Keyword: Stanozolol

1. Introduction

Since its rise to prominence in human athletics in the late 1980s and early 1990s, the synthetic anabolic steroid stanozolol (17α-methyl-5α-androstano-[3,2-*c*]-pyrazol-17β-ol) has become one of the most commonly used anabolic steroids in the Australian horseracing industry. While Australian rules of racing currently permit the use of registered veterinary anabolic steroids such as stanozolol out of competition, both they and their metabolites must have cleared the horse's system by the time it is presented to race. Hence, there is a requirement for appropriate doping control procedures.

The metabolism of stanozolol in humans has been investigated by a number of workers [1–3], mostly by electron ionization (EI) GC–MS analysis of pertrimethylsilylated urine extracts. Important metabolic pathways identified include hydroxylation at C3′, C4, C6 and C16, epimerization at C17 and combinations thereof (Fig. 1). Stanozolol itself is not generally detected. As a result of this work, a number of monohydroxystanozolols have become commercially available as forensic reference standards, and these now form the basis of stanozolol testing in human urine.

The only previously published study of the equine metabolism of stanozolol used atmospheric pressure chemical ionization (APCI) triple-quadrupole LC–MS to detect urinary metabolites hydroxylated at C3′, C4, C6 and C16 following oral administration [2] (Fig. 1). The study was fairly preliminary in nature however, and no attempt was made to elucidate either the stereochemistry of the observed metabolites or their phase II metabolism. Furthermore, the most common route of administration for stanozolol in Australian horses is by intramuscular injection of an aqueous suspension. Steroids so given are not subject to first pass hepatic metabolism and so may give rise to significantly different metabolic profiles than when administered orally [4]. Hence, this study was not considered ideal for our purposes.

Following further unpublished research at this and other laboratories, 16β-hydroxystanozolol was established as a major equine urinary metabolite of stanozolol following

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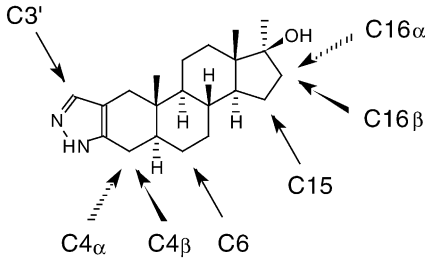

Fig. 1. Stanozolol and its known or proposed metabolic hydroxylation sites.

administration by intramuscular injection. Based on these findings, a GC–MS screen was developed to target this compound in equine urine [5]. The procedure involved a reversed phase–solid phase extraction followed by methanolysis of sulfate and β-glucuronide conjugates [6] and a series of liquid–liquid cleanup extractions. The dried residues were then acetonylated and analyzed by GC–MS with on-column methylation of the pyrazole function. While effective, this approach has proven to be very demanding in terms of time and labor and is obviously limited in targeting only one metabolite. Consequently, we have continued to investigate various means by which the scope of the screen could be broadened and its workload reduced.

Recently, a number of workers have reported the detection of stanozolol and/or 16β-hydroxystanozolol at parts per billion levels in bovine urine by electrospray ionization (ESI) ion trap LC–MS [7–9]. As this approach is applicable to a wide range of potential stanozolol metabolites with excellent sensitivity and with no requirement for chemical derivatization of the target analytes, we decided to investigate its applicability to equine urine. At the same time, we also took the opportunity to evaluate a commercially available enzyme-linked immunosorbent assay (ELISA) kit and to conduct a more thorough investigation of the equine phase I and phase II metabolism of stanozolol following administration by intramuscular injection.

2. Experimental

2.1. Chemicals and reagents

Pure stanozolol and stanozolol-$^2H_3$ (17α-trideutero-methyl-5α-androstano-[3,2-*c*]-pyrazol-17β-ol) were purchased from Sigma (Castle Hill, NSW, Australia), 3′-hydroxystanozolol from Radian (Austin, TX, USA), 4α-hydroxystanozolol from Alltech (Baulkham Hills, NSW, Australia) and 4β-hydroxystanozolol from the National Analytical Reference Laboratory (Pymble, NSW, Australia). 16β-Hydroxystanozolol was synthesized by B. Dent Global (Lower Hutt, New Zealand). *Escherichia Coli* β-glucuronidase enzyme was purchased from Roche Diagnostic (Castle Hill, NSW, Australia). Anhydrous methanolic hydrogen chloride (1 M) was prepared by dropwise addition of acetyl chloride (7 mL) to anhydrous methanol (100 mL) with stirring and cooling. All organic solvents were nanograde or better and were used without further purification.

2.2. Apparatus

Abselut Nexus solid phase extraction cartridges were purchased from Varian (Mulgrave, Vic, Australia) and Strata-X-C solid phase extraction cartridges from Phenomenex (Pennant Hills, NSW, Australia). Solid phase extractions were performed using Gilson (Villiers-le-Bel, France) ASPEC XL or XL4 liquid-handling systems. LC–MS analyses were performed using an Agilent (North Ryde, NSW, Australia) 1100 Series LC/MSD Ion Trap equipped with a Phenomenex (Pennant Hills, NSW, Australia) Synergi Hydro-RP 4 μm 1.0 mm id × 150 mm column and Optimize Technologies (Oregon City, OR, USA) Opti-Guard 40 μm 1.0 mm id × 15 mm guard column. Stanozolol ELISA kits (P/N SW2418) were purchased from Randox (Crumlin, UK) and processed with the aid of a Bio-Tek Instruments (Winooski, VT, USA) Model ELP 35 microplate washer and Sorin Biomedica (Saluggia, Italy) Eti-System microplate reader.

2.3. Animal administration

An aqueous suspension of stanozolol (Stanazol®, RWR Veterinary Products, Glenorie, NSW, Australia; 5 mL = 250 mg stanozolol) was administered to a 7-year old thoroughbred gelding (580 kg) by intramuscular injection. Urine samples were collected by conditioned spontaneous voiding daily for 28 days and immediately frozen and stored at −20 °C until required for analysis. The administration was approved by the New South Wales Thoroughbred Racing Board Animal Care and Ethics Committee.

2.4. looseness-1Extraction and deconjugation—phase I metabolic study

An aliquot of urine (3 mL) was adjusted to pH 7 and spiked with stanozolol-$^2H_3$ (3 ng) as an internal standard. The sample was centrifuged for 5 min at 3000 rpm to sediment particulate matter, after which the supernatant fraction was loaded onto an unconditioned Abselut Nexus solid phase extraction cartridge (30 mg, 1 mL). The cartridge was rinsed with sodium hydroxide solution (0.1 M, 1 mL) and water (1 mL), and then dried briefly with air. The steroids were eluted with methanol (1 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 hydrogen chloride (1 M, 0.5 mL) and incubating for 10 min at 60 °C. The reaction was quenched by the addition of sodium phosphate buffer solution (pH 8, 0.25 M, 2 mL), and the resulting solution was loaded onto an unconditioned Strata-X-C solid phase extraction cartridge (30 mg, 1 mL). The cartridge was washed with sodium hydroxide solution (0.1 M, 1 mL), hydrochloric acid (0.1 M,

1 mL), water (1 mL) and methanol (1 mL). After drying briefly with air, the target analytes were eluted with ethyl acetate:hexane:diethylamine (50:50:1, 1 mL). One-third of the eluate was set aside for analysis by ELISA and the remainder for LC–MS. Both aliquots were dried by evaporation at 80 °C under a stream of nitrogen.

2.5. Extraction and deconjugation—phase II metabolic study

An aliquot of urine (3 mL) was adjusted to pH 9.5 and unconjugated steroids were extracted with diisopropyl ether (3 mL). The remaining urine was then adjusted to pH 6.0 and incubated overnight at 37 °C with *E. Coli* β-D-glucuronidase enzyme concentrate (20 μL = approximately 90000 Fishman units). The urine was again adjusted to pH 9.5 and the freshly deglucuronidated steroids were extracted with diisopropyl ether (3 mL). Sulfate-conjugated steroids were then isolated by solid phase extraction of the residual urine and methanolyzed as described previously. Stanozolol-$^2H_3$ (3 ng) was added to each fraction as an internal standard, after which they were dried by evaporation at 80 °C under a stream of nitrogen.

2.6. LC–MS analysis

Dried residues were reconstituted in acetonitrile (25 μL) and formic acid (25 mM, 25 μL) for LC–MS analysis. Sample injections (8 μL) were made into an initial mobile phase of 20:80 acetonitrile:formic acid (25 mM). The acetonitrile content was held for 1 min, then increased linearly over 6 min to a final composition of 80:20 acetonitrile:formic acid (25 mM). This composition was held for a further 5 min. Total-column flow was 75 μL/min throughout and the column was maintained at a constant temperature of 40 °C. The MS interface was operated in positive ion electrospray ionization mode with a capillary voltage of +3.5 kV, nebulizer pressure of 25 psi, drying gas flow of 8 L/min and drying gas temperature of 350 °C. Ion optics, isolation and fragmentation parameters were individually optimized for a variety of target analytes and appear in Table 1. Ion charge control was set to 25000 and maximum accumulation time to 250 ms with averaging over two scans for a minimum scan rate of 2 s^{-1}. Data were acquired in full scan MS/MS mode over the range *m*/*z* 100–350.

2.7. Quantitation of stanozolol and 16β-hydroxystanozolol

Duplicate calibration curves were constructed for stanozolol and 16β-hydroxystanozolol in equine urine following the methods outlined previously for the phase I metabolic study and using stanozolol-$^2H_3$ (1 ng/mL) as an internal standard. Post-administration urine samples were also analyzed in duplicate. Chromatograms were constructed for the purpose of integration by extracting from the raw data the combined signals for *m*/*z* 121, 133, 149, 161, 175, 189, 203 and 229 for stanozolol, *m*/*z* 159, 175, 189, 201, 215, 227 and 255 for 16β-hydroxystanozolol, and *m*/*z* 124, 135, 149, 161, 175, 189, 203 and 232 for stanozolol-$^2H_3$. The curves were linear over the range 0–2 ng/mL with correlation coefficients (R) of 0.999 and 0.998, respectively. The limit of detection for both analytes was estimated to be around 0.1 ng/mL following the analysis of 10 different blank equine urine samples spiked at that concentration, all of which gave signal to noise ratios greater than 3:1.

2.8. ELISA analysis

Dried residues were reconstituted in the diluent buffer provided with the ELISA kit (200 μL) and analyzed as per the kit manufacturer's instructions.

3. Results and discussion

3.1. Extraction procedure

The extraction procedure developed here differs somewhat from conventional equine anabolic steroid extraction procedures in that it is based entirely on solid phase extraction techniques. This greatly facilitates the automation of the procedure with all the advantages in labor minimization and reproducibility that entails. That this may be possible is due largely to the use of two novel polymeric sorbents that have recently become available. Abselut Nexus (Varian) is a styrenedivinylbenzene based polymer with a hydrophilic functionality to increase its water wettability, functioning primarily as a reversed phase sorbent. Strata-X-C (Phenomenex) is similar, but also incorporates a benzenesulfonate function for mixed mode cation exchange capability.

Table 1
Ion optics and CID parameters for stanozolol and related compounds

Compound	Skimmer 1 (V)	Octopole (V)	Trap drive	Precursor (*m*/*z*)	Cut-off	Amplitude (V)
Stanozolol	55.2	2.48	32.8	329	100	1.4
Stanozolol-$^2H_3$	55.2	2.48	32.8	332	100	1.4
3′-Hydroxystanozolol	56.5	2.50	33.5	345	120	1.5
4α-Hydroxystanozolol	22.5	2.50	34.0	345	100	0.6
	41.5	2.46	32.0	309	90	1.1
4β-Hydroxystanozolol	22.5	2.50	34.0	345	100	0.6
	41.5	2.46	32.0	309	90	1.1
16β-Hydroxystanozolol	55.4	2.50	32.8	345	140	2.0

The use of Abselut Nexus for the extraction of anabolic steroids from equine urine was pioneered by Wynne et al. [10]. We have further taken advantage of the relative chemical inertness of the polymer by including an aqueous sodium hydroxide wash step prior to analyte elution. This wash was found to remove a large quantity of unwanted matrix material and led to significantly cleaner final extracts. The same wash was also employed during the second solid phase extraction to similar effect, and with both washes in place the need for any form of liquid–liquid cleanup extraction was removed.

A further purification of stanozolol and its metabolites was readily achieved using the ion exchange function of the Strata-X-C sorbent. This proved advantageous in two respects. Firstly, the pyrazole function of the stanozolol family renders them inherently unsuitable for GC–MS analysis, which is the analytical method of choice for most anabolic steroids. The separation based on basicity enables the stanozolol extracts to be obtained from our regular anabolic steroid screen for analysis by LC–MS or ELISA without the implementation of a whole new extraction procedure. Non-basic anabolic steroid metabolites if required may be eluted with ethyl acetate:hexane (50:50) prior to the methanol wash step and derivatized separately for GC–MS analysis.

Secondly, the extraction of stanozolol and its metabolites without taking advantage of the ion exchange purification led to serious problems with matrix interference during both LC–MS and ELISA analysis. In the former case, this was manifested through severe ion suppression effects in the electrospray ion source leading to greatly decreased sensitivity for the target analytes. In the latter, it resulted in an unacceptably high false positive rate, presumably due to antibody cross reactivity with endogenous steroidal material. With the ion exchange extraction in place, both of these problems were effectively overcome.

3.2. LC–MS analysis of stanozolol and monohydroxystanozolols

Under positive ion electrospray ionization conditions, stanozolol and its metabolites readily form stable proton adducts suitable for MS/MS analysis. However, collision-induced dissociation (CID) of these adducts in an ion trap was found to result in product ion mass spectra quite different to those obtained by CID in a triple-quadrupole instrument. This phenomenon is attributable to the inability of the ion trap to retain MS/MS product ions below the appropriate low-mass cut-off value, in our case ranging from *m*/*z* 90 to 140 depending on the precursor ion, and this prevented the capture of many important low-mass fragments readily observable in the quadrupole CID mass spectra of the compounds in question. The ion trap CID mass spectra thus appear distorted in favor of the high-mass end of the spectrum.

Mück and Henion [2] have described quadrupole CID mass spectra for stanozolol, 3′-hydroxystanozolol, 4β-hydroxystanozolol and 16α-hydroxy-17-epistanozolol, as well as a number of other human and equine monohydroxylated urinary stanozolol metabolites of unidentified stereochemistry. In general, they tend to be dominated by fragments at *m*/*z* 81 (stanozolol, 6-hydroxystanozolols and 16-hydroxystanozolols) or 97 (3′-hydroxystanozolols) derived from fission of the A-ring through C1/C10 and C3/C4. Notable exceptions are the 4-hydroxystanozolols, which dehydrate at C4/C5 to form a conjugated system, which stabilizes the A-ring against this particular fragmentation. The resulting-mass spectra are complex, displaying fragments corresponding to the breakdown of all five rings.

These results contrast sharply with the ion trap CID mass spectra of stanozolol and 16β-hydroxystanozolol published by De Brabander et al. [7]. Mück and Henion's intense *m*/*z* 81 peaks in each case are below the low-mass cut-off value and are not detected. Instead the higher mass fragments are emphasized, providing significant additional mass spectral information. We have now also subjected stanozolol-$^2H_3$, 3′-hydroxystanozolol, 4α-hydroxystanozolol and 4β-hydroxystanozolol to ion trap CID treatment, and the resulting product ion mass spectra appear in Fig. 2. All are significantly different to their quadrupole CID counterparts. LC retention data for all relevant compounds appear in Table 2.

Comparison of the ion trap CID mass spectra of stanozolol and stanozolol-$^2H_3$ shows the fragmentation of stanozolol to be highly complex, although many of the major ions may be explained as shown in Fig. 3. In general, fragments retaining the protonated pyrazole ring involve simple-ring fission only, while fragmentations involving loss of the pyrazole are typically accompanied by transfer of a single hydrogen to the neutral-loss fragment and dehydration of the hydroxyl at C17. This is exemplified by the base peak at *m*/*z* 121 (*m*/*z* 124 for stanozolol-$^2H_3$) deriving from fission of the C-ring through C8/C14 and C9/C11. Mück and Henion's A-ring fragment at *m*/*z* 81 is not observed, although the related fragmentation involving neutral loss of 4-methylpyrazole gives rise to an intense allyl stabilized fragment at *m*/*z* 229 (*m*/*z* 232 for stanozolol-$^2H_3$).

The ion trap CID mass spectrum of 3′-hydroxystanozolol is similar to that of stanozolol with the exception that fragments retaining the pyrazole ring are increased by *m*/*z* 16. There is no water loss fragment corresponding to the hydroxyl at C3′, presumably due to resonance stabilization of the C–O bond and the aromatic nature of the protonated pyrazole ring. This stabilization may also contribute to the base peak at *m*/*z* 229 through enhancement of the 3-hydroxy-

Table 2
Relative retention times for monohydroxystanozolols

Compound	RRT[a]
Proposed 15-hydroxystanozolol	0.58
Proposed 16α-hydroxystanozolol	0.67
3′-Hydroxystanozolol	0.76
4α-Hydroxystanozolol	0.78
4β-Hydroxystanozolol	0.83
16β-Hydroxystanozolol	0.82

[a] Relative to stanozolol.

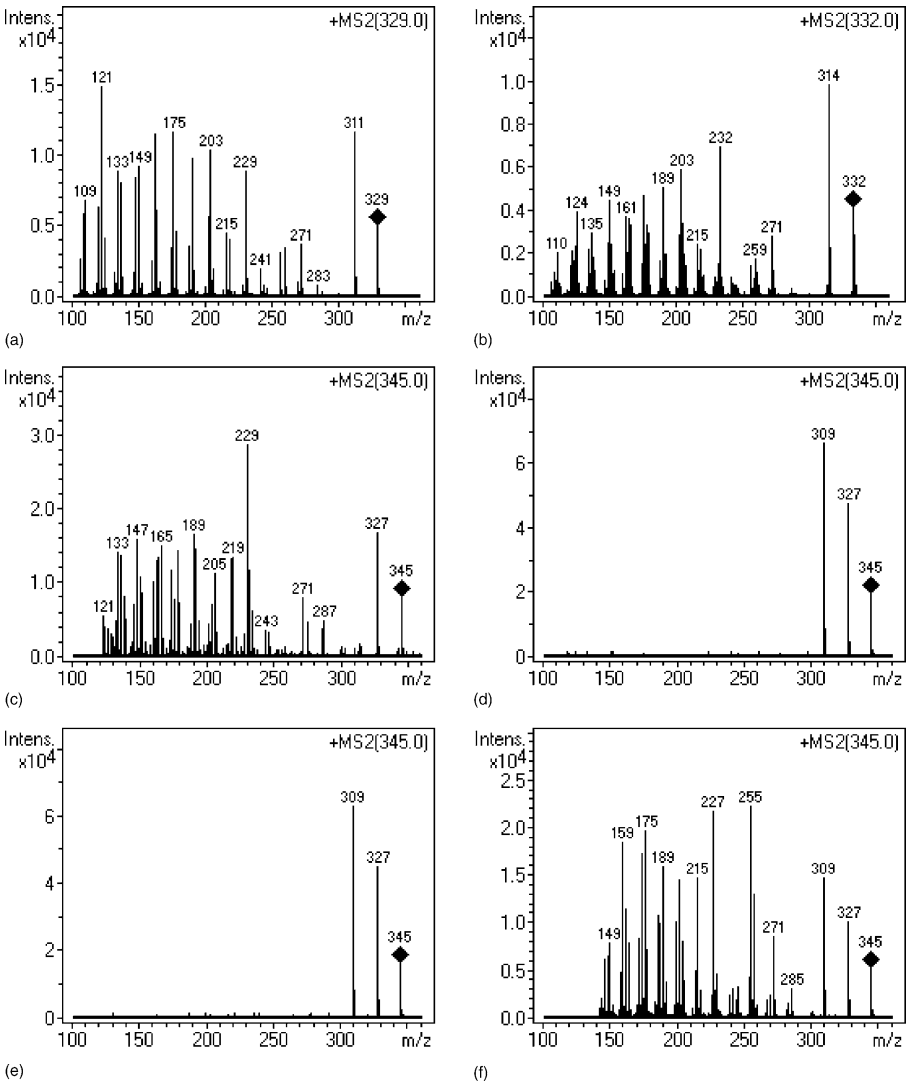

Fig. 2. Ion trap CID product ion mass spectra for proton adducts of (a) stanozolol, (b) stanozolol-$^2H_3$, (c) 3′-hydroxystanozolol, (d) 4α-hydroxystanozolol, (e) 4β-hydroxystanozolol and (f) 16β-hydroxystanozolol.

4-methylpyrazole neutral loss analogous to that already described for stanozolol.

4α-Hydroxystanozolol and 4β-hydroxystanozolol were found to be essentially identical by MS, with an LC retention time difference being their only distinguishing feature. Their ion trap CID mass spectra were very simple, consisting only of the precursor ion and dual water loss fragments at *m*/*z* 327 and 309. However, MS/MS/MS treatment using the *m*/*z* 309 fragment produced more detail, as shown in Fig. 4. These spectra are dominated by the base peak at *m*/*z* 145, which is also the base peak from quadrupole CID and was proposed by Mück and Henion to derive from fission of both C-ring and pyrazole ring together with an indeterminate loss of *m*/*z* 16. Based on our MS/MS/MS data, we would propose another possibility to be dehydration at C4 followed by B-ring fission through C6/C7 and C9/C10 with double-hydrogen transfer leading to the highly conjugated species shown in Fig. 5. The initial dehydrations at C4 and C17 were also readily achievable by ion source CID, and MS/MS treatment of the resulting *m*/*z* 309 fragments gave identical spectra to those shown in Fig. 4.

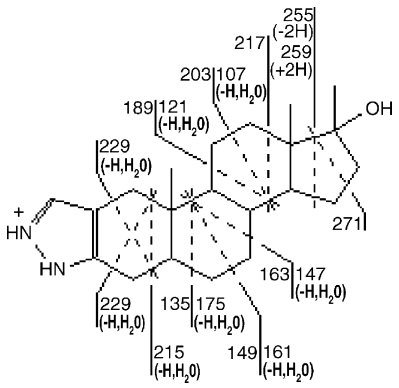

Fig. 3. Some proposed stanozolol fragmentations.

The ion trap CID mass spectrum of 16β-hydroxystanozolol is again similar to that of stanozolol but with

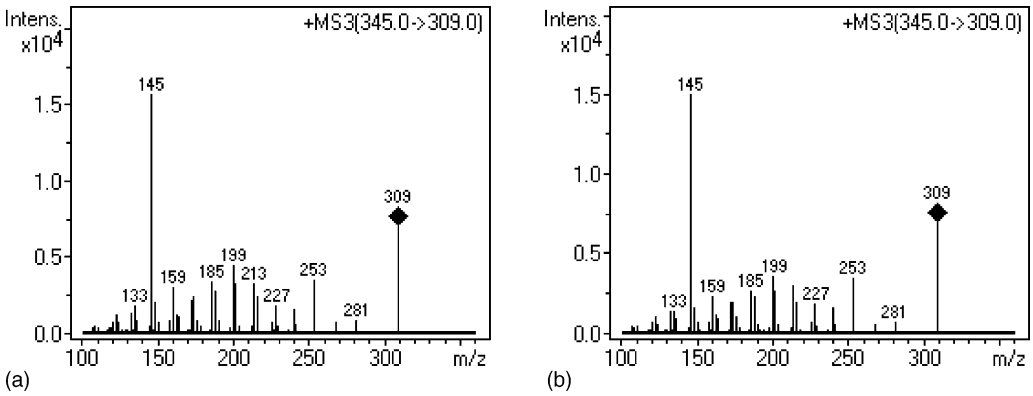

Fig. 4. Ion trap CID product ion mass spectra (MS/MS/MS) for proton adducts of (a) 4α-hydroxystanozolol and (b) 4β-hydroxystanozolol.

fragments retaining the D-ring being reduced by *m*/*z* 2, reflecting dehydration at C16. The *m*/*z* 255 fragment is also greatly increased in magnitude, becoming the new-base peak. This fragment is proposed to derive from D-ring fission through C13/C17 and C14/C15 and is presumably enhanced by the additional substituent at C16. The concurrent shift of the stanozolol *m*/*z* 259 fragment to *m*/*z* 257 may reflect a change in the final unsaturation patterns of this D-ring fragmentation, again due to the extra substituent at C16.

3.3. Phase I metabolism

After extraction and deconjugation, a total of four stanozolol related compounds were detectible in equine urine following the administration of a therapeutic dose of stanozolol by intramuscular injection. A typical LC chromatogram appears in Fig. 6. Of these, two were readily identifiable as stanozolol and 16β-hydroxystanozolol by comparison with authentic reference materials, and their excretion curves from day 1 to 28 appear in Fig. 7. Both compounds exhibit the "saw tooth" excretion pattern typical of long-acting anabolics in the horse [11–14] with a gradual rise in concentration over the first week followed by a plateau of about 1 week, then a gradual decline. Both compounds were detectible out to around day 26 with peak concentrations of 1.1 and 1.4 ng/mL, respectively. It should be noted however that subsequent experience has led us to believe that stanozolol excretion alone may not always be a reliable marker for stanozolol administration, as we have since identified two competition positive urine samples where hydroxylated stanozolol metabolites were unaccompanied by any detectible levels of the parent drug.

The remaining two metabolites were both identifiable as monohydroxystanozolols from their molecular weight and the appearance of water loss fragments in their CID mass spectra at *m*/*z* 327 and 309 as shown in Fig. 6. However, comparison with the full range of available reference materials could only confirm the hydroxylation sites as elsewhere than C3′ or C4. The mass spectrum of the later-eluting compound (peak 2) was almost identical to that of 16β-hydroxystanozolol, although it displayed a significantly shorter LC retention time. If it is allowed that the hydroxylation site is C16, there are four possible stereochemistries based on variation at C16 and C17. 16α-Hydroxylation and 17-epimerization have both been reported for stanozolol in humans [1,3], while in the horse they have been variously reported for the analogous anabolic steroids methandrostenolone [15–17], 17α-methyltestosterone [18], fluoxymesterone [19] and norethandrolone [20]. However, given that the accepted method of formation of 17-epimers from 17α-alkyl-17β-hydroxysteroids is via spontaneous hydrolysis of the tertiary 17β-sulfate conjugate in the urine [15], their formation is obviously critically dependant on the phase II metabolism. In the present case, the metabolite was demonstrated to exist in the urine as a mixture of sulfate and β-glucuronide conjugates. It is difficult to explain how the latter could produce a 17-epimerised artifact, and so it may be assumed that the deconjugated species observed during LC–MS analysis is not epimerized at C17. As the metabolite is clearly not 16β-hydroxystanozolol, we thus propose it to be 16α-hydroxystanozolol.

The earlier eluting unknown metabolite (peak 1) gave a CID mass spectrum not dissimilar to that of the 16-hydroxystanozolols, but with some distinct differences. That

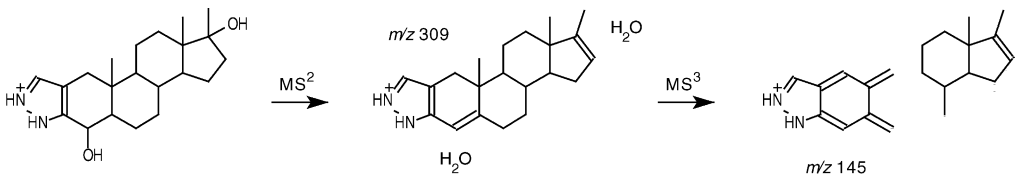

Fig. 5. Possible origin of the *m*/*z* 145 fragment formed from ion trap MS/MS/MS treatment of 4-hydroxystanozolols.

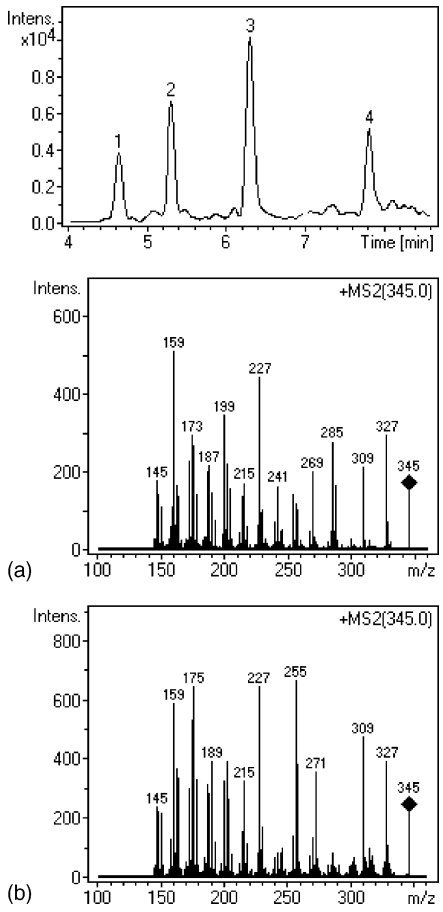

Fig. 6. Typical LC–MS-extracted ion chromatogram (*m*/*z* 159, 175, 189, 201, 215, 227 and 255 for 4–7 min and *m*/*z* 121, 133, 149, 161, 175, 189, 203 and 229 for 7–8.5 min) for stanozolol post-administration equine urine showing stanozolol (peak 4), 16β-hydroxystanozolol (peak 3) and two other unidentified monohydroxystanozolols (peaks 1 and 2) together with ion trap CID product ion mass spectra for (a) peak 1 and (b) peak 2.

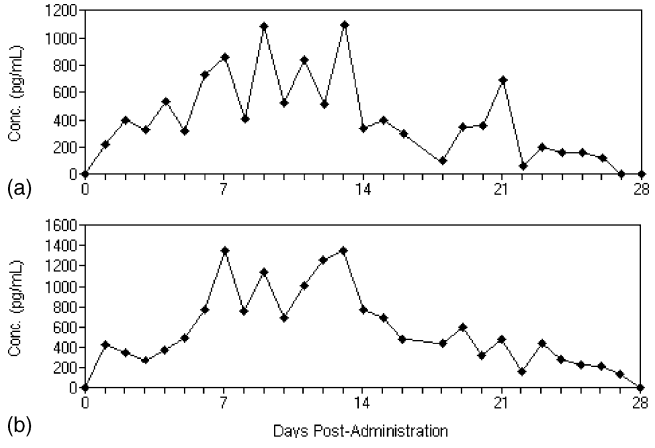

Fig. 7. Equine urinary excretion curves for (a) stanozolol and (b) 16β-hydroxystanozolol following the intramuscular injection of 250 mg stanozolol.

it is not a 16-hydroxystanozolol is evidenced by the shift of the stanozolol/16-hydroxystanozolol *m*/*z* 271 fragment corresponding to D-ring fission through C13/C17 and C15/C16 to *m*/*z* 269, indicating dehydration of a hydroxyl elsewhere than C16, C17 or the 17-methyl substituent. However, the retention of the stanozolol/16-hydroxystanozolol fragments at *m*/*z* 255, 257 and 259 corresponding to D-ring fission through C13/C17 and C14/C15 indicate the extra hydroxylation site to be at one of the latter sites or C15. The obvious inference then is a 15-hydroxystanozolol, and 15-hydroxylation has previously been proposed as an equine metabolic pathway for 17α-methyltestosterone [21]. This is also consistent with the base peak at *m*/*z* 159 deriving from B-ring fission through C6/C7 and C9/C10 together with hydrogen transfer and double dehydration. However, more research would be required to enable a definite assignment. As with the proposed 16α-hydroxy metabolite, the compound was present in the urine as a mixture of sulfate and β-glucuronide conjugates, and it is therefore assumed that it is not a 17-epimer.

Both of the newly observed metabolites showed similar excretion patterns to stanozolol and 16β-hydroxystanozolol at comparable concentrations, although accurate quantitation was not possible without the appropriate reference materials. 3′-Hydroxystanozolol, 4α-hydroxystanozolol and 4β-hydroxystanozolol were searched for but not detected. A preliminary search was also conducted for dihydroxylated metabolites, previously reported in humans [1–3], but these again were not detected.

3.4. Phase II metabolism

A knowledge of the phase II metabolism of anabolic steroids is critical in devising analytical techniques for their detection in urine. In the literature, the most commonly reported phase II metabolic processes affecting anabolic steroids in the horse and other species are conjugation by β-glucuronidation and sulfation. β-Glucuronides may be effectively hydrolyzed using any of a number of commercially available β-glucuronidase enzymes. However, steroidal sulfate conjugates are not always hydrolyzed by commercially available β-glucuronidase/arlysulfatase mixed enzyme preparations, instead requiring some form of acid solvolysis for efficient cleavage [22,23]. In the present study, sulfate and β-glucuronide conjugates were fractionated by treatment with β-glucuronidase from *E. Coli*, which readily hydrolyzes the β-glucuronides but has no appreciable sulfatase activity. The residual sulfates could then be isolated and methanolyzed separately.

For stanozolol, all four compounds detected in the urine were excreted as a mixture of sulfate and β-glucuronide conjugates with little or no unconjugated material detected. Stanozolol and the proposed 15-hydroxy metabolite were predominantly β-glucuronidated, while the 16α- and 16β-hydroxy metabolites were mostly present as sulfates. The approximate ratios of sulfate to glucuronide were 1:5, 1:3,

4:1 and 2:1, respectively. There was no major variation in the phase II metabolism over time.

3.5. ELISA results

Enzyme-linked immunosorbent assay is a relatively modern technique enabling rapid and highly specific testing for target analytes in biological matrices. In the context of a large-scale drug screening operation, its main attractions are speed, simplicity and a low false positive rate. As a part of this study, we evaluated a commercially available stanozolol ELISA kit (Randox) for possible use as a preliminary-screening tool for stanozolol in equine urine samples.

The kit concerned claims a limit of detection for stanozolol in human urine of 0.6 ng/mL with 40% cross reactivity for 16β-hydroxystanozolol. The possibility of cross reactivity with other stanozolol metabolites must also be allowed, and hence its overall sensitivity may well be significantly greater than implied by its sensitivity for stanozolol alone. Indeed, if the primary epitope is at the pyrazole end of the molecule, it may be particularly well suited to equine stanozolol analysis where the major metabolites are all modified in relatively remote positions.

When trialed with untreated or enzyme-hydrolyzed equine urine, the ELISA kit was found to be completely ineffective. When the samples were extracted, methanolyzed and cleaned up with either a single liquid–liquid extraction or a simple reversed phase–solid phase extraction a false positive rate of around 80% (n = 20) was observed, which was obviously still unacceptable. Introduction of the mixed mode cation exchange solid phase extraction with an ethyl acetate–hexane wash prior to the basic elution step brought the false-positive rate down to around 40% (n = 20), but usable results were only obtained after the substitution of methanol for the final-wash step. This protocol resulted in adequate ELISA suppression for suspect sample identification out to at least day 28 for the stanozolol post-administration samples and a false-positive rate of less than 1% (n = approximately 2000 to date). An ELISA based excretion curve for the post-administration samples showing suppression relative to the negative control sample appears in Fig. 8. For routine analysis of competition samples, an equine urine sample spiked to 1 ng/mL with both stanozolol and 16β-hydroxystanozolol is run with each batch to provide a cut-off level for suspect samples. Unfortunately, a more detailed quantitative evaluation was not possible without appropriate reference materials.

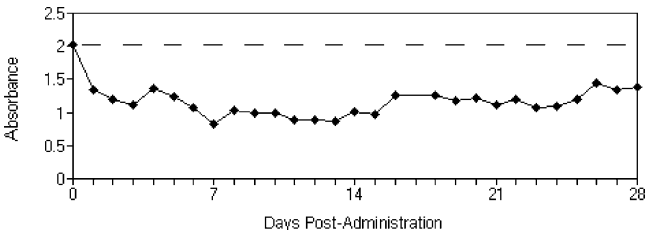

Fig. 8. ELISA-based equine urinary excretion curve showing suppression relative to the negative control sample following the intramuscular injection of 250 mg stanozolol. The analyses were performed on methanolyzed urine extracts.

4. Conclusion

The administration of an aqueous suspension of stanozolol to the horse resulted in the excretion of stanozolol, 16β-hydroxystanozolol and two other monohydroxylated metabolites tentatively proposed as 16α-hydroxystanozolol and a 15-hydroxystanozolol. Phase II metabolism in the form of sulfate and β-glucuronide conjugation was extensive. The excretion of all four compounds was typically erratic over a period of about 4 weeks with peak concentrations in the 1–2 ng/mL range for a therapeutic dose of stanozolol. Positive-ion electrospray ionization ion trap LC–MS provided a sensitive and specific means of testing for these substances in equine urine, with limits of detection around 0.1 ng/mL and mass spectra containing large numbers of diagnostic ions. Enzyme-linked immunosorbent assay provided a convenient alternative for large-scale preliminary screening.

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