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17ß-HYDROXY-3-OXOANDROST-4-EN-19-OIC ACID AS A SOURCE OF NANDROLONE IN ACID SOLVOLYSED STALLION URINE EXTRACTS

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ABSTRACT

At the 15th ICRAV in Dubai, evidence was presented that certain 19-norsteroids typically observed in acid solvolysed stallion urine extracts may actually be chemical artefacts formed through the spontaneous decarboxylation of endogenous steroid-19-oic acids. In the case of the nandrolone frequently detected in such samples, this explanation provides an alternative to the traditional assumption that nandrolone sulphate and/or nandrolone glucuronide are normal components of stallion urine. This, in turn, raises the possibility of using intact phase II conjugates of nandrolone as markers for exogenous nandrolone administration.

A previously reported component of stallion urine, 17ß-hydroxy-3-oxoandrost-4-en-19-oic acid (testosterone-19-oic acid), was synthesised and its chemical properties were examined. It was found to be very labile under acidic conditions, losing carbon dioxide to give free nandrolone, but was relatively stable at neutral or basic pH. SPE and LC/MS/MS procedures were developed for both nandrolone-17-sulphate and testosterone-19-oic acid in urine, and a survey of 30 entire male equine urine samples indicated that, although most contained significant concentrations of the latter, none contained any detectable levels of the former. All 30 samples showed nandrolone when analysed by GC/MS after SPE and acid solvolysis using anhydrous methanolic hydrogen chloride.

INTRODUCTION

The definitive early work on nandrolone metabolism in the horse was carried out at the Horseracing Forensic Laboratory (HFL) some 20–30 years ago (Houghton 1977; Houghton and Dumasia 1980; Dumasia and Houghton 1984). These studies identified a number of important phase I biotransformations, including reduction of the Δ^4-3-ketone function, oxidation and epimerisation of the 17ß-hydroxyl function and oxygenation at C16. Phase II metabolism was found principally to involve the sulphation of 17ß-hydroxylated metabolites and ß-glucuronidation of 17α-hydroxylated metabolites. Ultimately, nandrolone-17-sulphate, 5α-estrane-3ß,17ß-diol-17-sulphate and 5α-estrane-3ß,17α-diol-17-ß-glucuronide (Fig 1) were proposed as the major metabolites derived from the exogenous administration of nandrolone esters to the horse (Teale and Houghton 1991), and these compounds now form the basis of nandrolone screening in female and gelded male horses. For ease of analysis, the analytes of interest are typically subjected to enzymolysis and/or acid solvolysis to eliminate phase II conjugation prior to chemical derivatisation and GC/MS analysis.

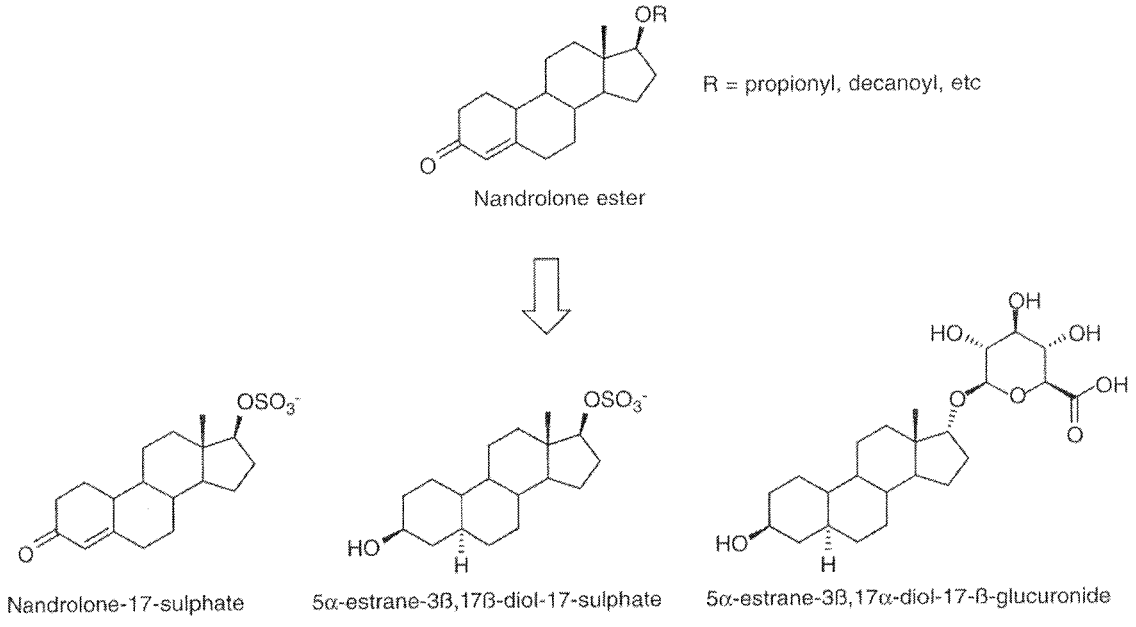

Fig 1: The principal equine metabolites of nandrolone esters given by im injection.

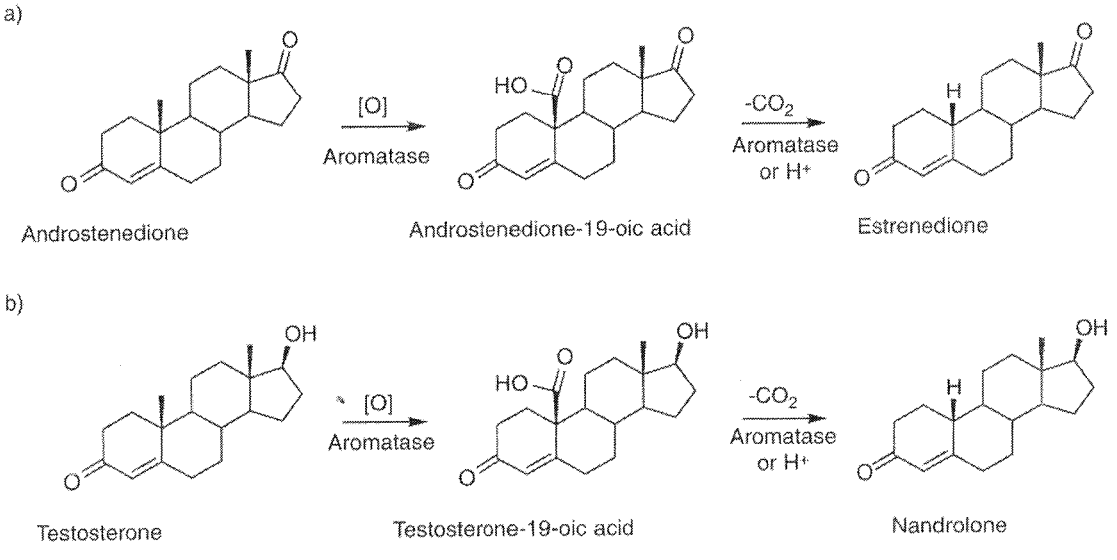

Fig 2: The proposed origins of: a) estrenedione; and b) nandrolone from their respective C_{19} analogues.

The situation in the entire male horse was found to be more complicated, however. SPE and acid solvolysis of stallion urine commonly produces a residue containing both nandrolone and 5α-estrane-3ß,17α-diol, as well as other neutral C_{18} steroids such as estr-4-ene-3,17-dione (Houghton *et al.* 1984). The appearance of nandrolone in the sulphoconjugated fraction was generally attributed to the presence of endogenous nandrolone-17-sulphate in the urine, which would obviously negate this compound as a simple qualitative marker for exogenous nandrolone administration. Attempts to establish a suitable nandrolone threshold level for reporting purposes were unsuccessful, and consequently the criterion established for the detection of nandrolone administration in the entire male horse is based on the ratio of free and conjugated 5α-estrane-3ß,17α-diol, a known phase I metabolite of nandrolone, to free and conjugated

estr-5(10)-ene-3ß,17α-diol, an endogenous C_{18} steroid which is not a metabolic product of nandrolone.

Dumasia *et al.* (1989) found homogenised equine testicular tissue to contain 19-hydroxysteroids in addition to 19-norsteroids, and proposed them to be intermediates in the bioconversion of androgens to estrogens. Subsequently, Garrett *et al.* (1991) observed that porcine granulosa cells *in vitro* could convert androst-4-ene-3,17-dione to 3,17-dioxoandrost-4-en-19-oic acid (androstenedione-19-oic acid), and thence to estr-4-ene-3,17-dione (Fig 2a). Houghton *et al.* (2005) have further developed this work with the identification of androstenedione-19-oic acid and, more importantly, testosterone-19-oic acid in stallion urine. Based on these findings, it was proposed that the source of nandrolone in acid solvolysed stallion urine extracts may not in fact be nandrolone-17-sulphate after all, but rather testosterone-19-oic acid (Fig 2b).

The implications of these findings for the science of nandrolone screening in the entire male horse are significant. If it is allowed that the nandrolone observed in acid solvolysed stallion urine extracts is principally a chemical artefact of testosterone-19-oic acid, then it is quite possible that little or no endogenous nandrolone-17-sulphate is actually present. As nandrolone-17-sulphate is a known metabolite of nandrolone esters in the horse, the possibility of using it as a marker for exogenous nandrolone administration in the entire male horse then becomes apparent. To further our understanding of the matter, we have now synthesised testosterone-19-oic acid and examined its chemical properties under various conditions. Furthermore, we have developed methods for the direct LC/MS detection of nandrolone-17-sulphate and testosterone-19-oic acid in equine urine, and have applied the same to a collection of entire male equine urine samples. The results of this work are presented below.

Materials and methods

Chemicals and equipment

19-hydroxy-3-oxoandrost-4-en-17ß-yl acetate (19-hydroxytestosterone-17-acetate) was purchased from Steraloids (Rhode Island, USA). Nandrolone-17-sulphate and nandrolone-16,16,17-$^2H_3$-17-sulphate were purchased from the National Measurement Institute (New South Wales, Australia). Oasis WAX SPE cartridges were purchased from Waters (New South Wales, Australia) and Abselut Nexus SPE cartridges from Varian (Victoria, Australia). Hydroxyammonium chloride was purchased from Aldrich (New South Wales, Australia) and *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) from UCT (Pennsylvania, USA).

Synthesis of testosterone-19-oic acid

Testosterone-19-oic acid was prepared from 19-hydroxytestosterone-17-acetate in 66% yield by Jones oxidation and base hydrolysis. Full experimental details and characterisation data will be published elsewhere.

Sample preparation method 1

Aliquots of urine (2 ml) were adjusted to pH 7 and spiked with nandrolone-16,16,17-$^2H_3$-17-sulphate (200 ng) as an internal standard, then were loaded onto Oasis WAX SPE cartridges (3 ml, 60 mg) which had previously been conditioned with methanol (2 ml) and water (2 ml). The cartridges were rinsed with water (3 ml) and methanol (3 ml) and eluted with ethyl acetate:methanol: diethylamine (45:5:1 v/v/v; 3 ml). The eluates were evaporated to dryness at 70°C under nitrogen, then were reconstituted in methanol (10 µl) and water (90 µl) for LC/MS analysis.

Sample preparation method 2

Aliquots of urine (2 ml) were adjusted to pH 7 and spiked with nandrolone-16,16,17-$^2H_3$-17-sulphate (200 ng) as an internal standard, then were loaded onto Abselut Nexus SPE cartridges (3 ml, 60 mg) which had previously been conditioned with methanol (2 ml) and water 2 ml). The cartridges were rinsed with water (3 ml) and eluted with methanol (3 ml). The eluates were evaporated to dryness at 70°C under nitrogen, then were reconstituted in anhydrous methanolic hydrogen chloride methanol (0.5 ml) and incubated for 10 min at 60°C. Aqueous sodium hydroxide (2 M, 4 ml) was added and the solvolysed steroids were extracted into diisopropyl ether (4 ml). The extracts were evaporated to dryness at 70°C under nitrogen. The residues were reconstituted in a solution of hydroxyammonium chloride in pyridine (10% w/v, 50 µl) and incubated for 30 min at 80°C. *N,N*-Dimethylformamide and MTBSTFA were then added and incubated for a further 60 min at 80°C. Water (2 ml) was added and the derivatised steroids were extracted into hexane (4 ml). The extracts were evaporated to dryness at 70°C under nitrogen and reconstituted in dodecane (100 µl) for GC/MS analysis.

TABLE 1: MS/MS parameters for LC/MS analysis

Analyte	Isolation		Fragmentation	
	Mass (m/z)	Width (m/z)	Cut-off (m/z)	Energy (V)
Testosterone-19-oic acid	319	1.2	120	0.90
Nandrolone-17-sulphate	355	1.2	100	0.65
Nandrolone-16,16,17-$^{2}H_3$-17-sulphate	358	1.2	100	0.65

Sample preparation method 3

Aliquots of urine (2 ml) were adjusted to pH 7 and washed with diisopropyl ether (2 x 4 ml) to remove non-acidic steroids such as free nandrolone. They were then spiked with nandrolone-16,16,17-$^{2}H_3$-17-sulphate (200 ng) as an internal standard, and were loaded onto Oasis WAX SPE cartridges (3 ml, 60 mg) which had previously been conditioned with methanol (2 ml) and water (2 ml). The cartridges were rinsed with water (3 ml), then were eluted first with formic acid in methanol (3% v/v, 3 ml) and secondly with diethylamine in methanol (3% v/v; 3 ml). Both eluates were evaporated to dryness at 70°C under nitrogen, and were then subjected to methanolysis and derivatised for GC/MS analysis as described for Method 2 above.

LC/MS analysis

LC/MS data were acquired on an Agilent (New South Wales, Australia) 1100 Series LC/MSD Ion Trap instrument equipped with a Phenomenex (New South Wales Australia) Synergi Hydro-RP column (150 mm x 1 mm ID) and an Optimize Technologies (Oregon, USA) Opti-Guard C18 guard column (15 mm x 1 mm ID). Sample injections (8 µl) were made into an initial mobile phase of 95% aqueous ammonium acetate (25 mM) and 5% acetonitrile. After holding for 1 min, the organic content was ramped in a linear gradient to 90% total composition at 15 min. This composition was held for a further 4 min, then was returned to the starting point and equilibrated for 5 min prior to the next injection. A flow rate of 70 µl/min and column temperature of 40°C were maintained throughout. The MS was operated in positive ion electrospray ionisation mode with a capillary voltage of 3.5 kV, nebuliser pressure of 40 psi, drying gas flow of 8 l/min and drying temperature of 350°C. MS parameters were optimised for the individual analytes. Data were collected in full scan product ion mode over the range m/z 100–400. MS/MS parameters for all analytes appear in Table 1.

GC/MS analysis

GC/MS data were acquired on an Agilent (Victoria, Australia) 6890 GC/5973 MSD instrument equipped with a SGE (Victoria, Australia) BPX-5 capillary column (30 m x 0.25 mm ID, 0.25 µm film thickness) and using helium as carrier gas. Sample injections (2 µl) were made in splitless mode with an injector temperature of 260°C. The column was initially held at 100°C for 1 min, then was ramped at 20°C/min to 300°C and held for a further 9 min. Head pressure was programmed to maintain a constant flow rate of 1 ml/min, with the purge valve opening at 2 min to a split of 500:1. The MS was operated in electron ionisation mode with a transfer line temperature of 280°C. Data were collected in SIM mode using the target ions m/z 460 for nandrolone and 463 for nandrolone-16,16,17-$^{2}H_3$. The dwell time for each target was 10 ms.

Results and Discussion

Testosterone-19-oic acid was prepared from 19-hydroxytestosterone-17-acetate by a Jones oxidation followed by base hydrolysis of the acetate protecting group. Its stability in aqueous solution was examined over a broad pH range, and as may be predicted from its structure, it was found to be quite labile below pH 5, undergoing a facile decarboxylation to yield free nandrolone. The rate of conversion was pH dependent, with complete conversion requiring about 48 h at pH 1 and twice that at pH 3. In contrast, treatment with anhydrous methanolic hydrogen chloride at 60°C caused complete decarboxylation in minutes. In light of these observations it is curious that the compound can be synthesised using such a strongly acidic concoction as Jones reagent, and this has not yet been explained. However, there is no doubt that under standard methanolysis conditions as used in many routine equine anabolic screening methods, testosterone-19-oic acid will be comprehensively converted to nandrolone. At neutral or basic pH, testosterone-19-oic acid was somewhat less labile, appearing to degrade over a period of weeks rather than days.

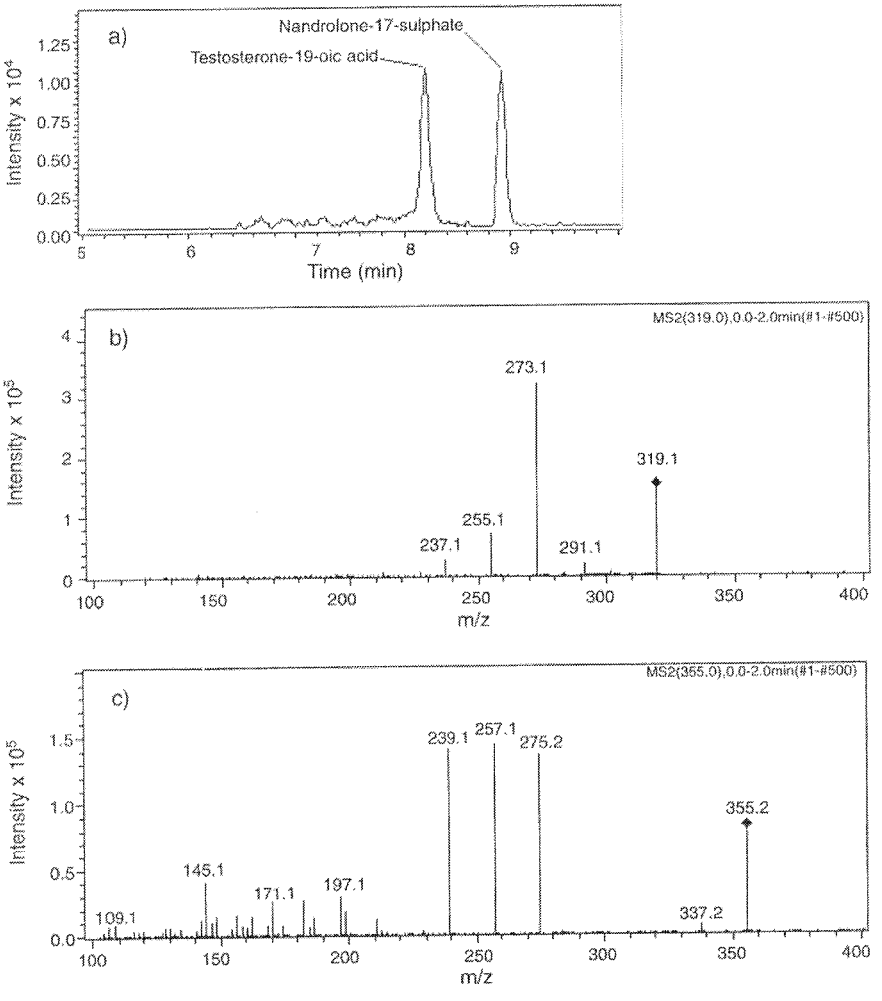

Fig 3: a) Extracted ion chromatograms for nandrolone-17-sulphate and testosterone-19-oic acid in a 30 ng/ml urine spike; b) positive ion full scan ion trap MS/MS spectrum for nandrolone-17-sulphate; c) positive ion full scan ion trap MS/MS spectrum for testosterone-19-oic acid.

To enable a direct comparison of testosterone-19-oic acid and nandrolone-17-sulphate levels in entire male equine urine, a SPE-LC/MS method was developed for the simultaneous analysis of both compounds (Method 1). This was achieved using Oasis WAX, a polymeric mixed mode weak anion exchange sorbent, and the residues were analysed without further purification. The 2 analytes were well separated by LC (Fig 3a), and MS analysis was carried out in positive ion electrospray ionisation mode, which was found to give superior overall results to negative ion mode. Under positive ion conditions, both analytes gave proton adducts, which in turn gave rise to useful product ion spectra (Figs 3b, 3c). The principal fragment in the MS/MS spectrum of testosterone-19-oic acid (m/z 273) presumably derives from a loss of the C19 carboxyl group as formic acid, while the nandrolone-17-sulphate MS/MS spectrum is dominated by fragments deriving from the loss of sulphur trioxide (m/z 275) and sulphuric acid (m/z 257) and a fragmentation thought to involve dehydration and aromatisation of the A-ring (m/z 239). Under negative ion conditions, testosterone-19-oic acid gave a carbon dioxide loss fragment at m/z 273 as the highest mass peak, with slightly lower overall sensitivity in MS/MS mode. Nandrolone-17-sulphate showed a strong peak at m/z 353, but MS/MS treatment of this precursor gave very poor sensitivity, thought to be because the expected hydrogen sulphate fragment at m/z 97 lies below the low mass cut-off of the ion trap and is not detected. Consequently, triple quadrupole MS may be the preferred option for this compound in negative ion mode.

On application to equine urine samples, the LOD for nandrolone-17-sulphate was estimated as being around 15 ng/ml based on a signal to noise ratio greater than 3. Of 30 competition entire male equine urine samples tested by this method, none showed any indication of nandrolone-17-sulphate, while 24 showed varying concentrations of testosterone-19-oic acid. On GC/MS analysis following conventional SPE and methanolysis steps (Method 2), all 30 samples showed the presence of nandrolone. The appearance of nandrolone in 6 samples which showed neither nandrolone-17-

sulphate nor testosterone-19-oic acid by LC/MS is attributed to the comparatively higher sensitivity of the GC/MS method (about 20-fold) and/or the breakdown of testosterone-19-oic acid to free nandrolone in the urine prior to analysis.

Given the sensitivity limitations of the LC/MS method in detecting nandrolone-17-sulphate directly, an additional GC/MS experiment was designed to compare levels of nandrolone-17-sulphate and testosterone-19-oic acid indirectly (Method 3). Non-acidic steroids were first removed by a solvent extraction at neutral pH, after which steroidal sulphates and carboxylic acids were resolved by SPE using the Oasis WAX sorbent. Elution with acid modified methanol provided a fraction containing the carboxylic acids, while subsequent elution with base modified methanol provided a sulphate conjugated fraction. After conventional methanolysis and derivatisation steps, both fractions were analysed by GC/MS. In each case, nandrolone was detected in the carboxylic acid fraction only, indicating that the original source of the nandrolone is not a sulphate conjugate. As the nandrolone LOD for the GC/MS procedure is in the order of 1 ng/ml, this strongly suggests that the actual concentration of nandrolone-17-sulphate in the urine is considerably less even than the 15 ng/ml allowed by the LC/MS procedure.

These results strongly suggest that nandrolone-17-sulphate is not a normal component of entire male equine urine at concentrations in the ng/ml range, and that testosterone-19-oic acid is a more likely precursor for the nandrolone commonly observed in acid solvolysed stallion urine extracts. Given that nandrolone-17-sulphate is a known metabolite of nandrolone esters in the horse, it should therefore be considered as a potential marker for exogenous nandrolone administration in the entire male horse. In continuation of this work, we intend to examine ways of improving the LOD for the direct detection of nandrolone-17-sulphate in equine urine with a view to conducting a broader survey to establish whether there is any basal nandrolone-17-sulphate concentration in entire male horses.

Conclusion

Testosterone-19-oic acid was synthesised and was shown to be only partially stable in urine over a period of weeks at neutral or basic pH. It was considerably less stable under acidic conditions, and was extremely labile under standard methanolysis conditions. The principal decomposition product formed in each case was free nandrolone. Methods for the direct detection of both nandrolone-17-sulphate and testosterone-19-oic acid in equine urine were developed and applied to a selection of 30 entire male equine urine samples. The presence of testosterone-19-oic acid was confirmed directly in 24 samples and indirectly in the remainder. Nandrolone-17-sulphate was not detectible either directly or indirectly in any samples, and therefore may be useful as a marker for the detection of exogenous nandrolone administration in entire male horses.

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