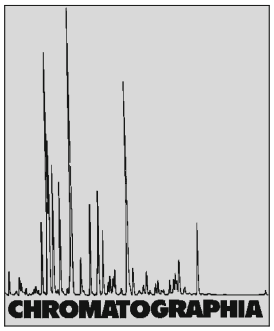
Validation of an Analytical Biomarker Approach for the Detection of Nandrolone Abuse in the Porcine



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Abstract

The use of anabolic steroids as growth promoting agents in food production is prohibited under European Union legislation, but there is currently no internationally accepted method for detecting the abuse of the anabolic steroid nandrolone in the porcine. Therefore, an analytical biomarker approach based on gas chromatography-tandem mass spectrometry (GC-MS-MS) analysis of the major urinary free fraction nandrolone metabolite 19-noretiocholanolone was developed and validated. The lower and upper limits of quantification of the assay were 25 and 3,000 pg mL^{-1} respectively. The limit of detection was calculated as 13.2 pg mL^{-1}, which is significantly lower than previously reported methods. When applied to a population of untreated animals, 19-noretiocholanolone distributions in boars and gilt were bimodal, with a small number of concentrations in each sex at around the 1,000 pg mL^{-1} region and the majority of concentrations closer to the lower end of the calibration range. Statistical analysis of the data was carried out in order to suggest screening and confirmatory threshold approaches for this steroid in the urine of boars and gilts. The adopting of particular screening thresholds would be at the discretion of the individual regulating authorities, but at a false non-compliance rate of 1 in 10,000 of the normal population, the suggested confirmatory thresholds (7,501.6 pg mL^{-1} for boars and 19,200.4 pg mL^{-1} in gilts) are able to detect the abuse of nandrolone for several weeks following administration of this steroid.

Keywords

Gas chromatography–tandem mass spectrometry
Threshold
Porcine
Pig
Steroids
Nandrolone

Introduction

The use of anabolic steroids as growth promoting agents in food production is prohibited under European Union legislation [1]. However, some androgenic–anabolic steroids, such as testosterone (17β-hydroxy-androst-4-en-3-one), nandrolone (17β-hydroxy-estr-4-en-3-one), and boldenone (17β-hydroxy-androsta-1,4-dien-3-one), are known to be endogenous in certain species [2], making a simple qualitative determination of their presence insufficient for proving abuse. The detection of intact steroid esters or the use of combustion isotope ratio mass spectrometry can be useful for the confirmation of certain endogenous steroids [2], but these methods are not suitable for all steroids and are too laborious to be used as screening approaches. Therefore, a quantitative threshold or biomarker approach is usually required to regulate the use of endogenous steroids.

For many years, nandrolone was considered to be a purely synthetic steroid, but in the 1980s it was isolated as a natural hormone in the stallion [3] and boar [4]. Since then, nandrolone

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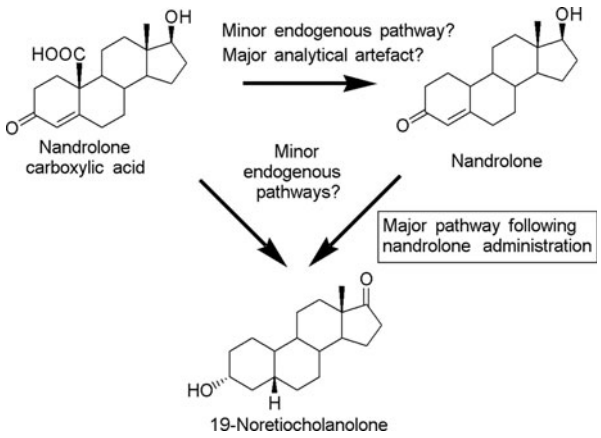

Fig. 1. Diagram depicting some of the possible metabolic pathways leading to the presence of 19-noretiocholanolone in porcine urine. A similar pathway could also operate starting with androstenedione carboxylic acid in place of nandrolone carboxylic acid, leading to either 19-noretiocholanolone or 19-norandrostenedione as metabolites

related compounds have also been detected in the bovine [5], ovine [6], caprine [7], human [8] and cervine [9]. The presence of nandrolone in boar meat also has potential consequences for consumers, since it has been shown that following consumption of boar meat, human urine contains increased concentrations of nandrolone metabolites [10].

Studies on the metabolism of nandrolone following administration of the steroid [11, 12] have shown that nandrolone itself is excreted in urine predominantly as a sulphate, while a number of a-ring reduced metabolite isomers are present in the free, glucuronide and sulphate fractions. Although nandrolone has been found to be present in the urine and several other matrices of untreated porcine animals [13–15], these studies have reported that the a-ring reduced metabolites were not detected in porcine urine.

The absence of a-ring reduced metabolite in the 'natural' state is hard to rationalise if nandrolone is truly produced by the porcine, since nandrolone administration studies have clearly demonstrated them to be major metabolites. A clue to the real source of nandrolone in porcine urine may lie in studies carried out in the equine, where similarly to the porcine, nandrolone is detected in the urine of intact males, but the a-ring reduced metabolites are much less abundant than would be expected if nandrolone itself were present [16]. Nandrolone in the equine is now known to be predominantly an analytical artefact of the breakdown of a 19-carboxylic acid precursor that is endogenous in this species [16]. It has therefore recently been suggested [2] that such 19-carboxylic acids may also be the precursors to urinary nandrolone in the porcine (Fig. 1). This theory is supported by studies in porcine granulosa cells, where the existence of a 19-carboxylic acid derivative of androstenedione has been demonstrated [17].

Since the a-ring reduced nandrolone metabolites are present in large quantities in the urine of treated, but not untreated porcine animals, a quantitative method for 19-noretiocholanolone (3α-hydroxy-5β-androstan-17-one) (Fig. 1) in the urinary free fraction was therefore validated and applied to real samples in order to produce thresholds for screening and/or confirmation of nandrolone abuse. The reason for choosing 19-noretiocholanolone in particular is because it has been shown to be one of the most abundant urinary metabolites following nandrolone administration and was therefore predicted to be a good biomarker of nandrolone abuse [11]. Analysis of the free fraction was preferred over the glucuronic or sulphate fractions because the free fraction contains the highest concentrations of 19-noretiocholanolone [11] and also because the other fractions contain significant quantities of other materials that interfere in GC–MS based analytical assays (James Scarth, personal observation).

Experimental

Chemicals and Reagents

Deionized water was prepared using an SG Ultrachem TWF UV system (Barsbuttel, Germany). All organic solvents, buffers and bases were analytical grade and were purchased from Fisher Scientific (Loughborough, UK). Ammonium iodide, *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA), dimethylformamide (DMF) and ethane thiol were obtained from Sigma-Aldrich (Dorset, UK). C18, 6 cc, 500 mg and silica, 6 cc, 500 mg solid phase extraction cartridges were supplied by Waters (Wexford, Ireland). 19-noretiocholanolone was supplied by Steraloids (Rhode Island, USA) and D_4-19-noretiocholanolone was provided by LGC Promochem (Teddington, UK).

Collection of Samples from Untreated Animals

Urine samples were obtained in the UK from both live animals (various university and research institute owned herds; from animals housed in metabolic cages and collecting urine using a floor-based drainage system) and from animals going for slaughter (organically farmed animals slaughtered for food production; collecting urine directly from the bladder post-mortem). A total of 205 gilt urine samples (none from pregnant animals) were obtained from animals ranging from 10 to 39 weeks of age and a total of 263 boar urine samples were obtained from animals ranging from 10 to 28 weeks of age. Whilst future European legislation may lead to a more widespread banning of porcine castration, many countries in Europe currently prefer to rear castrated males because of the potentially unsavoury 'boar taint' odour of meat from intact males [18]. Boar taint is believed to result from the increased concentrations of skatole and

androstenone produced by intact boars [18]. However, the rearing of intact males is currently preferred in the UK because these animals are generally slaughtered earlier than in Europe, with the result that the animals are less likely to reach sexual maturity and hence produce lower concentrations of skatole and androstenone. Also, reducing profit margins in the UK pork industry have meant that the economic benefits to rearing intact males are also factors in refraining from castration (various researchers and organic farming consultants—personal communications 2008 and 2009). Because of the current UK practice to rear intact males, no urine samples from castrated males were available for study.

Samples were collected and stored chilled on ice within 1-h. Samples on ice were then delivered by courier within 48-h before being frozen at −80 °C until the time of analysis.

Preparation of Calibration Lines and QCs

Calibration lines were constructed using pooled urine from castrated male bovine animals as a surrogate matrix. Bovine calibrant urine was augmented with 19-noretiocholanolone at concentrations of 0, 25 (the lower limit of quantification or LLOQ), 50, 100, 250, 500, 1,000, 1,500, 2,000 and 3,000 (the upper limit of quantification or ULOQ) pg mL^{-1}. The reason for not using pooled porcine urine for constructing calibration lines was twofold; firstly because it contained measurable endogenous concentrations of 19-noretiocholanolone (bovine urine did not) and secondly because the volumes needed to produce enough of the pool for both the validation and sample analysis studies were not available.

In order to validate the use of bovine urine as a surrogate calibration line matrix, pooled porcine boar urine was used as the QC matrix. Porcine QC concentrations were; the endogenous concentration only (E), endogenous augmented with 25 pg mL^{-1} (E + 25), 100 pg mL^{-1} (E + 100), 1,000 pg mL^{-1} (E + 1,000) and 2,000 pg mL^{-1} (E + 2,000).

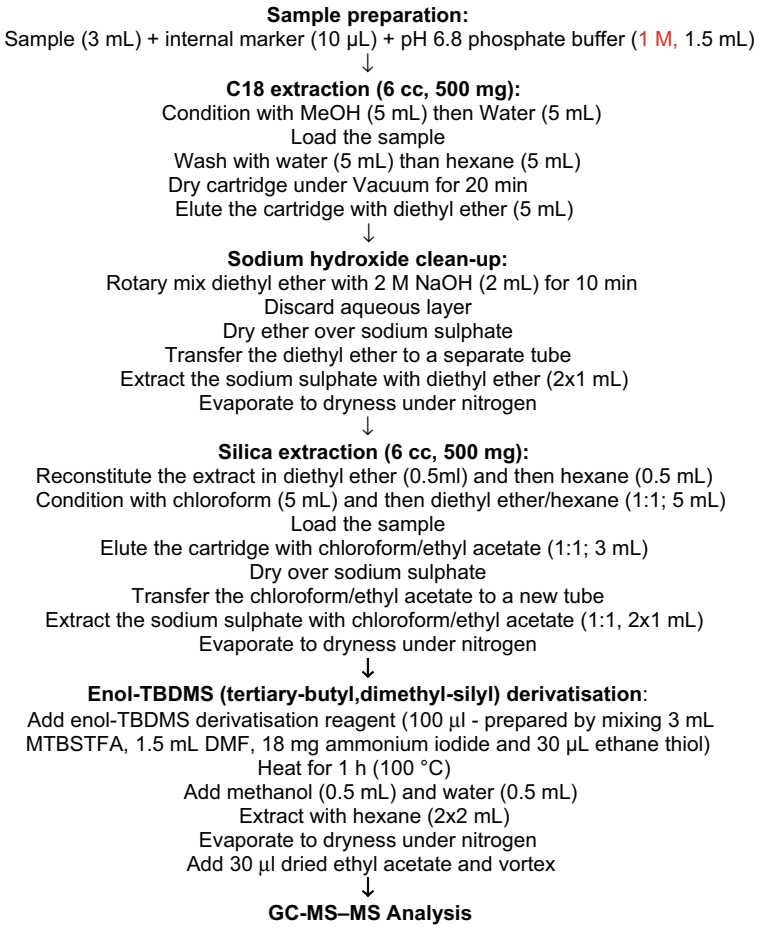

Fig. 2. Schematic of the extraction method

Extraction Method

Samples were prepared and extracted according to the scheme shown in Fig. 2. The D_4-19-noretiocholanolone internal standard was added at a concentration of 800 pg mL^{-1} to all samples (other than an extracted blank bovine urine control sample).

Method Validation Protocol

The validation protocol was based on the US Food and Drug Administration (FDA) Centre for Drug Evaluation and Research guidelines for bioanalytical method validation [19], with accuracy defined as the degree of relative error (% RE) and precision as the coefficient of variation (% CV). In terms %RE and %CV acceptance limits, the FDA recommended ± 20% limit for the lower limit of quantification (LLOQ) and ± 15% limit for all other concentrations were relaxed to 20 and 25% respectively. This was because the assay was considerably more complex than the majority of small molecule applications that the FDA guidelines are designed for. These somewhat relaxed limits were considered 'fit-for-purpose' according to the recommendations for biomarkers of Lee et al. [20], the results of which are taken into account through the application of uncertainty of measurement (see later section on statistical analysis). Three separate precision and accuracy batches were extracted and analyzed in order to determine the inter-batch precision and accuracy, chromatographic separation, selectivity, linearity, lower and upper limits of quantification (LLOQ and ULOQ respectively) and the limit of detection (LOD). Each precision and accuracy batch contained a blank

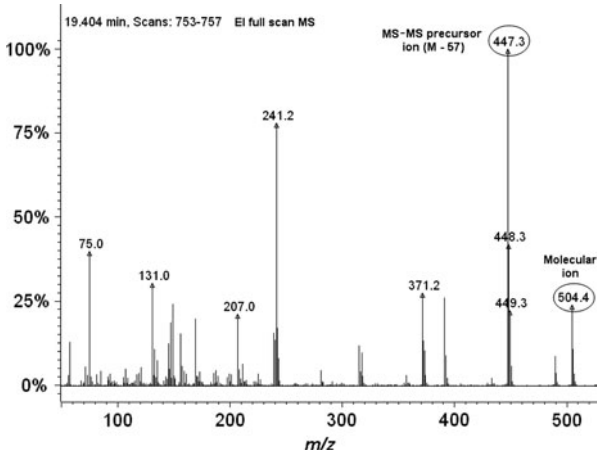

Fig. 3. Full scan EI-MS spectrum of 19-noretiocholanolone-di-TBDMS (tertiary-butyl,dimethyl-silyl) at 20 eV, highlighting the molecular ion and the fragment chosen as the MS/MS precursor ion

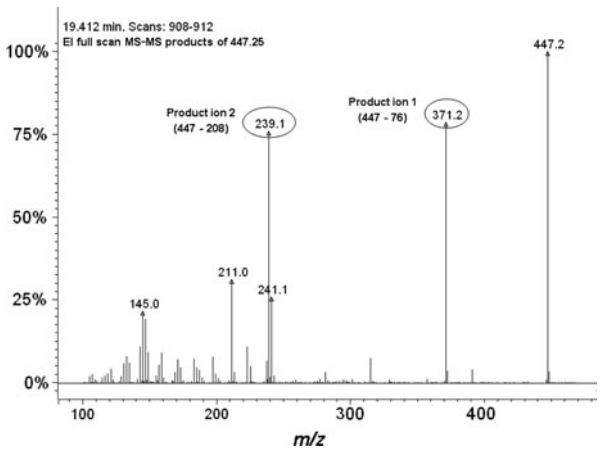

Fig. 4. Full scan EI-MS–MS spectrum of 19-noretiocholanolone-di-TBDMS at a collision energy of 7 eV, highlighting the MS–MS product ions

sample (extracted bovine urine with no internal standard or analyte added), a bovine urine calibration line (as described in the above section) and five replicates of each of the porcine urine QC concentrations (E, E + 25, E + 100, E + 1,000 and E + 2,000 pg mL^{-1}).

Stability of the analytes in porcine the E + 100 and E + 1,000 porcine QCs was assessed by measuring 19-noretiocholanolone concentrations at 3-, 6-, 9-, 12 and 13.5-months (the longest period for which 'real' samples were stored) and comparing the results to those determined at time-zero.

Recovery was assessed by comparing porcine urine spiked with 19-noretiocholanolone at 1,000 pg mL^{-1} before (matrix fortified) and after (matrix matched) extraction ($n = 5$).

The ability to dilute from outside the calibration range was assessed by spiking pooled porcine urine with 16,667 pg mL^{-1} 19-noretiocholanolone and then performing a 1 in 10 dilution with pooled porcine urine in order to bring the resulting concentration of analyte into the workable calibration range.

Analysis of Samples from Untreated Animals

Following validation of the method, samples from untreated animals were analyzed alongside a bovine urine spiked calibration line (25–3,000 pg mL^{-1}, made up of nine separate calibrant concentrations) and six porcine urine spiked QCs (two at each of the E + 100, E + 1,000 and E + 2,000 pg mL^{-1} concentrations) interspersed with the samples throughout the batch.

GC–MS–MS Method

Nine microlitres of sample was introduced into a Varian 1079 programmable temperature vaporiser (PTV) injector held at 125 °C for 1 min and then ramped to 280 °C at a rate of 150 °C min^{-1} before being held at 280 °C for 13 min. The injector split/column flow ratio was 20:1 until 1 min, before being reduced to 0:1 until 3 min, before being increased to 50:1 for the remainder of the run-time. Chromatography was carried out using a Varian CP-3800 gas chromatograph with helium as the carrier gas (1.0 mL min^{-1}) and a 30 m × 250 μm × 0.25 μm Varian VF-17MS column initially held at 120 °C for 2.00 min and then ramped by 50 °C min^{-1} to 220 °C at 4.00 min, ramped by a further 4.5 °C min^{-1} to 291 °C at 19.78 min, held for 2.00 min, ramped by a further 4.5 °C min^{-1} to 300 °C at 23.78 and then ramped by 50 °C min^{-1} to 320 °C at 28.18 min.

Mass spectrometry was carried out using a Varian 320-MS triple quadrupole mass spectrometer operated in the electron ionization (EI) mode. Transfer line temperature was 280 °C, ion source temperature was 220 °C, electron energy was 20 eV and electron multiplier voltage was typically around 1,800 V. Positive ion MS–MS data was acquired in the selected reaction monitoring (SRM) mode using argon as the collision gas at a pressure of 1.5 m Torr. Precursor and parent m/z transitions monitored were 451.3–375.2 for D_4-19-noretiocholanolone (collision energy 7 V), 447.3–371.2 for 19-noretiocholanolone transition 1 (used for quantification) (collision energy 7 V) and 447.3–239.1 for 19-noretiocholanolone transition 2 (used to provide additional support for the peak of interest relating to 19-noretiocholanolone) (collision energy 10 V) (Figs. 3, 4). Dwell times were 0.167 s for each transition. Data were acquired and processed using

the Varian Workstation version 6.9 software.

Statistical Analysis of the Untreated Animal Population Data

In order to control the abuse of nandrolone in the porcine, the statistical analysis set out to suggest 19-noretiocholanolone thresholds with various probabilities (1 in 20, 1 in 100, 1 in 1,000 and 1 in 10,000) of finding a larger value by chance in a natural population. The one-tailed version of the Chebyshev inequality was used. This method, described by Estler [21], is based on probability theory and makes minimal assumptions about the distribution of the data. Chebyshev confidence intervals are as far as possible from a mean for a given standard deviation; they are distribution-independent confidence intervals and are ideally suited to dealing with non-normally distributed data such as those resulting from the current study. A one-tailed Chebyshev confidence interval is given by

$$p \leq \frac{1}{1 + t^2/V}$$

Hence

$$t \leq \sqrt{\frac{V}{p} - V}$$

Where:

t is the difference between the mean and concentration at the upper confidence interval
V is the variance (square of the standard deviation),
p is the probability that difference between the mean concentration and the concentration of a sample taken at random is greater than t.

To assess the extent to which concentration differed between gilts and boars, a Monte Carlo permutation test was implemented [22]. Here, gender was permuted amongst individuals and the mean difference in concentration of 19-noretiocholanolone between gilts and boar was calculated for each permutation. The observed difference was then compared to the distribution of mean differences for 1,000 permutation and significance assessed on the basis of the position of the observed mean in the distribution.

Uncertainty of measurement was calculated using a Monte Carlo sample [22] from the distribution consistent with the observed result along with the analytical performance observed at validation. 1,000 samples were taken from these distributions and 1,000 Chebyshev threshold estimates were generated. The different probabilities of finding a value by chance in a 'natural' population are then reported at the upper 95% quantile results after factoring in the determined method uncertainty. Values for CCα and CCβ (decision limit and detection capability respectively) on each Chebyshev threshold associated with these probabilities [23], again at the 95% confidence interval, were also calculated for each analytical method used.

Results

In the following section all raw data and calculated threshold concentrations are uncensored and are given to one decimal place. All other summary statistics are presented to three significant figures.

Chromatography

Complete chromatographic separation for 19-noretiocholanolone in bovine urine was achieved (Fig. 5). In some porcine urine samples there was a small peak close after 19-noretiocholanolone (Fig. 5), but this did not reach significant enough a size to interfere with the integration of 19-noretiocholanolone.

Selectivity

In addition to the chromatographic separation, selectivity was assessed through monitoring the ratio of two separate MS–MS transitions for 19-noretiocholanolone, which support the suggestion that the peaks found in the porcine samples relate to 19-noretiocholanolone (Figs. 4, 5).

Linearity

The calibration curves (weighted $1/x$) were linear over the range studied (25 to 3,000 pg mL^{-1}—see chromatograms in Fig. 5). The mean r^2, slope, intercept and response factor % RSD values were 0.995, 0.00143, 0.0150 and 14.8 respectively ($n = 15$ over the validation and sample analysis phases).

Lower Limits of Quantification and Detection

The limit of quantification (25 pg mL^{-1}) was defined by the lowest point on the bovine urine calibration curve that could be quantified with a relative error of less than 25% (based on the FDA guidelines, as discussed earlier). The LOD was calculated using the endogenous porcine QCs analyzed during the validation. The mean S:N of the 19-noretiocholanolone peaks in these samples was 8.15 at a mean calculated concentration of 35.9 pg mL^{-1}. When defining the LOD as the concentration that leads to a S:N of 3:1, extrapolation of the endogenous porcine QC data leads to a predicted LOD of 13.2 pg mL^{-1}.

Precision and Accuracy

Inter-batch precision and accuracy during both the validation and sample analysis stages were good, with relative errors (%RE) and relative standard deviations (RSD) within 15.0% throughout (Tables 1, 2).

Linearity of Dilution

Results of QC samples diluted 1 in 10 from above the calibration range were acceptable, with the relative error and RSD being less than 2% in each case (Table 3).

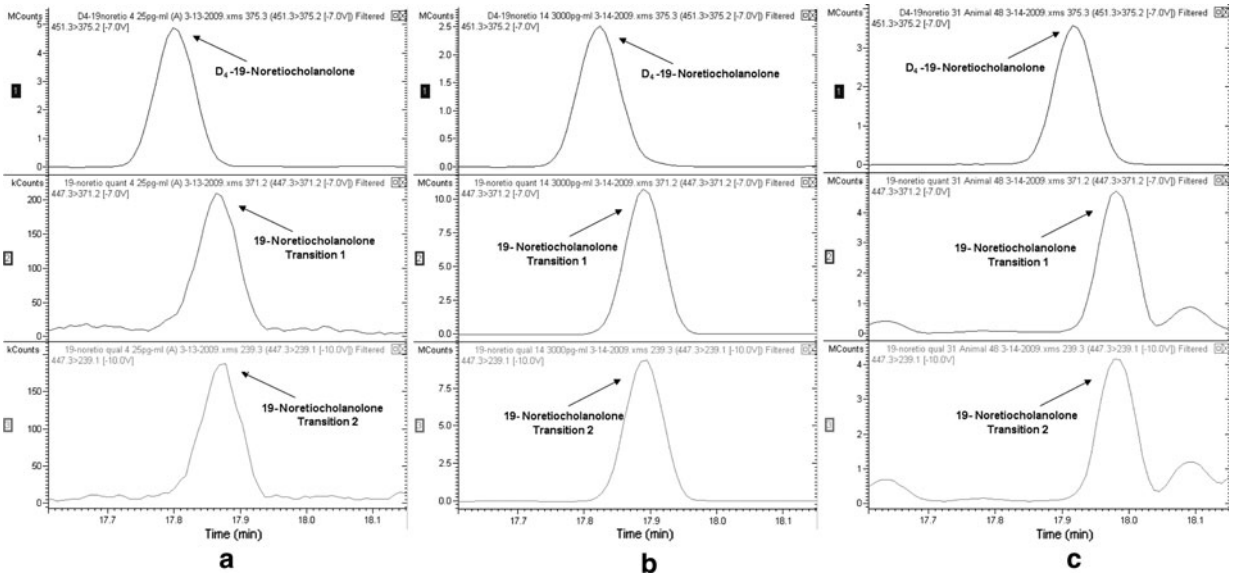

Fig. 5. Chromatograms obtained for samples at **a** the lower limit of quantification (a 25 pg mL^{-1} spiked calibration line sample. Note: this peak was absent in unspiked bovine urine), **b** the upper limit of quantification (a 3,000 pg mL^{-1} spiked calibration line sample) and **c** a porcine urine from the sampled population with a determined concentration of 896.5 pg mL^{-1}. The top trace refers to the D4-19-noretiocholanolone internal standard (m/z 451–375), the middle trace refers to 19-noretiocholanolone transition 1 (used for quantification) (m/z 447–371) and the bottom trace refers to 19-noretiocholanolone transition 2 (used to provide additional support for the peak of interest relating to 19-noretiocholanolone) (m/z 447–239)

Table 1. Inter-batch precision and accuracy results for 19-noretiocholanolone concentrations in porcine QCs during the validation ($n = 15$ at each concentration)

	Endogenous QCs pg mL^{-1} (E)	Endogenous + 25 pg mL^{-1} QCs (E + 25)	Endogenous + 100 pg mL^{-1} QCs (E + 100)	Endogenous + 1,000 pg mL^{-1} QCs (E + 1,000)	Endogenous + 2,000 pg mL^{-1} QCs (E + 2,000)
Theoretical conc.[a]	36.1	61.1	136.1	1,036.1	2,036.1
Measured mean conc.	35.9	68.6	136.4	1,049.8	1,945.9
SD	5.22	10.1	16.1	56.6	147
% RSD	14.5	14.7	11.8	5.39	7.55
% RE	−0.391	12.3	0.237	1.32	−4.43

[a] The theoretical concentration for the endogenous QCs was the mean of the determined concentrations in the endogenous QCs in the first of the three precision and accuracy validation batches. The theoretical concentration for the remainder of the QCs was this endogenous concentration plus the known spiked amount on top of this number i.e. endogenous + 100 pg mL^{-1} for LOW QCs

Table 2. Inter-batch precision and accuracy results for 19-noretiocholanolone concentrations in porcine QCs during the sample analysis phase of the study ($n = 24$ at each concentration)

	Endogenous + 100 pg mL^{-1} QCs (E + 100)	Endogenous + 1,000 pg mL^{-1} QCs (E + 1,000)	Endogenous + 2,000 pg mL^{-1} QCs (E + 2,000)
Theoretical conc.[a]	136.1	1,036.1	2,036.1
Measured mean conc.	141.0	1,036.3	2,021.0
SD	15.0	134	320
% RSD	10.6	12.9	15.8
% RE	3.61	0.0186	−0.743

[a] See footnote to Table 1

Recovery

The mean recovery of 19-noretiocholanolone spiked at 1,000 pg mL^{-1} ($n = 5$ for each of the matrix fortified and matrix matched spikes) was 87.4%.

Matrix Stability

19-noretiocholanolone was found to be stable in urine at −80 °C during the 13.5-months period studied (within ± 20.0% of time-zero values at all subsequent time-points studied).

Application of the Method to a Population of Samples from Untreated Animals

The descriptive statistics of the population data from untreated animals are shown in Table 4. 19-Noretiocholanolone distributions in boars and gilt were bimodal, with a small number of concentrations in each sex at around the 1,000 pg mL^{-1} region and the majority of concentrations closer to the lower end of the calibration range. The ratio of the two MS–MS transitions in these samples is consistent with them relating to 19-noretiocholanolone (Fig. 5).

Statistical Analyses of the Population Data

19-Noretiocholanolone concentrations in gilt and boar urines were determined to be statistically significantly different from each other (Monte Carlo permutation test $p < 0.025$, 1,000 permutations) and hence threshold calculations were performed separately for each sex. The estimated thresholds of finding a value by chance in a 'natural' population at different probabilities are given in Table 5 and are reported as the '95% confidence interval,' signifying that they are the upper estimates for the thresholds after factoring in the method's uncertainty of measurement. Table 5 also lists the CCα and CCβ values relating to these thresholds when using the analytical methodology presented herein. The application of the resulting thresholds for screening and confirmation of nandrolone abuse will be discussed in the following section.

Table 3. Results of the 1 in 10 dilution QCs (note: no samples needed to be diluted during the population sample analysis phase)

	Measured concentration (pg mL^{-1}) after 1 in 10 dilution	Adjusted concentration (relative to the theoretically spiked concentration of 16,667 pg mL^{-1})[a]
Measured mean conc.	1,679.4	16,399.4
SD	28.9	289
% RSD	1.72	1.76
% RE	N/A	−1.61

[a] Because of the endogenous contribution in the porcine urine used for dilution, in order to compare the relative error of the measured concentrations in the diluted samples compared to the theoretically spiked concentration, it was necessary to adjust the measured values for the endogenous contribution. This was achieved by subtracting the endogenous concentration of the diluting matrix (as measured by the mean of five endogenous QCs run in this batch) from the dilution QC measured value and then multiplying the result by the dilution factor of 10

Table 4. Summary statistics for determined 19-noretiocholanolone concentrations in the free fraction of porcine urine

	Boars	Gilts
Mean concentration (pg mL^{-1})	12.2	40.5
SD	69.7	183
% RSD	572	452
Minimum determined concentration (pg mL^{-1})	<LOD	<LOD
Maximum determined concentration (pg mL^{-1})	937.2	1181.8
n	263	205

Table 5. The 95% quantile thresholds (the upper threshold estimate after factoring in uncertainty of measurement) and associated CCα/β concentrations ($p = 0.05$) for free fraction 19-noretiocholanolone in porcine urine at different false non-compliance probability rates

	Chebyshev thresholds (pg mL^{-1}) at different false non-compliance probability rates			
	1 in 20	1 in 100	1 in 1,000	1 in 10,000
Boar				
95% quantile	345.4	763.8	2,384.3	7,501.6
CCα	396.4	876.4	2,735.8	8,607.5
CCβ	464.9	1,027.9	3,208.9	10,096.0
Gilt				
95% quantile	885.1	1,955.8	6,103.4	19,200.4
CCα	1,015.6	2,244.2	7,003.2	22,031.1
CCβ	1,191.2	2,632.2	8,214.2	25,840.8

Discussion

Previous studies have suggested the existence of endogenous nandrolone in porcine urine and at the same time the absence of the a-ring reduced metabolites in untreated animals [11–15]. Several authors have therefore suggested that 19-noretiocholanolone could be used as a biomarker for nandrolone abuse [2, 11, 12]. The results of the current study have shown that while concentrations of 19-noretiocholanolone are relatively low (maximum of 0.937 and 1.182 ng mL^{-1} in boars and gilts respectively), it has been demonstrated

for the first time that 19-noretiocholanolone is endogenous in this species. Since the limit of detection (13.2 pg mL^{-1}) was much lower than previously reported methods (due to the analysis of the urinary 'free' fraction, the use of a PTV injector and analysis by GC–MS–MS), it is not surprising that a small amount of 19-noretiocholanolone can be detected in porcine urine, since it might be expected that a small proportion of the suggested nandrolone carboxylic acid precursors might be converted into nandrolone metabolites in vivo.

The endogenous nature of 19-noretiocholanolone, albeit at low concentrations, means that a threshold approach rather than a simple qualitative demonstration of it's presence is required in order to be able to use this compound as a biomarker of nandrolone abuse. The principle of adopting thresholds to control the abuse of endogenous compounds has long been an accepted approach in both food production [24] and animal sports [25]. If used to formally confirm the abuse of an endogenous substance, the standard approach is to set the threshold at a statistical probability of finding a false non-compliance at a rate of 1 in 10,000 in a natural population [25], since this is considered to offer a sufficiently large safety margin to prevent the occurrence of false non-compliances.

Thresholds for screening may be set at a lower probability, but there then needs to be a secondary mechanism for confirmation if the confirmatory threshold is not also breeched. Typically, this may include follow-up analyses using gas chromatography carbon isotope mass spectrometry (GC-C-IRMS) [26], detection of an intact steroid ester [27] or an on-farm inspection (Jack Kay, personal observation). In an ideal world, a confirmatory threshold would also be suitable as a screening threshold, but this requires that the threshold is able to produce both low rates of false compliance and non-compliance; an ideal that is seldom achieved.

The analytical method presented herein was validated to a high standard and therefore considered suitable for application to a population of animals for use in establishing urinary 19-noretiocholanolone confirmatory thresholds. Because of the non-normal distribution of the data, it was necessary to use a non-parametric method of statistical analysis and the Chebyshev was considered the most suitable as it makes minimal assumptions about the population distribution and produces conservative thresholds relative to methods based on normally distributed data (James Scarth, personal observation).

As shown in Table 5, the suggested confirmatory thresholds for free fraction 19-noretiocholanolone concentrations at a false non-compliance probability rate of 1 in 10,000 are 7.502 and 19.200 ng mL^{-1} for boars and gilts respectively. To put these thresholds into context, in a recent study administering 2 mg kg^{-1} nandrolone laurate via intra-muscular injection to six boars aged 8–10 weeks, the mean free fraction 19-noretiocholanolone concentration at the last time-point of the study (15 days following administration) was 28.4 ng mL^{-1}, with a range of 9.6–53.6 ng mL^{-1} [12]. The suggested threshold of 7.502 ng mL^{-1} in boar urine should therefore be able to detect the abuse of nandrolone for a significant time period in most treated animals and lead to rates of both low false compliance and non-compliance.

Quantitative data on 19-noretiocholanolone concentrations following nandrolone administration to gilts are lacking, but if the results are similar to boars, then the suggested threshold of 19.200 ng mL^{-1} in gilt urine should still be able to detect abuse in a significant number of cases in this sex also. Since no castrated males were available within the current study, it was not possible to suggest thresholds for these animals, but if the current (2009) UK trend to leave animals intact continues, then a threshold for castrated animals would not be required.

Although the current study has focussed on male and female porcine animals, recent studies have also suggested that inter-sex porcine animals may occasionally be encountered [28, 29]. Therefore, it is important that these animals be identified during regulatory surveillance procedures in order that they can either be excluded from measurement against threshold concentrations or be subject to further investigations to rule out nandrolone abuse should a breech of one of the threshold concentrations be observed.

Threshold concentrations at false non-compliance probabilities of lower than 1 in 10,000 are also given in Table 5 in order that these concentrations could also be used in screening programmes prior to confirmatory analysis using one or more of the alternative follow-up techniques described earlier, should this be required. For example, should screening thresholds be set at a false non-compliance probability of 1 in 1,000 instead of the 1 in 10,000 used for confirmation, then the resulting threshold concentrations for boars and gilts are reduced by a factor of approximately threefold to 2.384 and 6.103 ng mL^{-1} respectively. With reference to the 19-noretiocholanolone concentrations reported in the Ventura et al. 2008 nandrolone administration study, these threshold concentrations would be able to detect 100% of the instances of nandrolone use in boars at the last time point studied (15 days), while also leading to a low rate of follow-up work (1 in 1,000 samples tested). Ultimately, the decision to establish a particular screening threshold is at the discretion of the individual regulatory authority and will no doubt reflect the striking of a balance between the amount of unnecessary follow-up work generate and the ability to minimize false non-compliances at the different probabilities. Although the CCα and CCβ values for the thresholds using the method above are also given in Table 5, these are unique to this laboratory. If other laboratories adopt these thresholds, then CCα and CCβ values must be independently determined in these laboratories before applying the method to 'real-life' samples.

In conclusion, the analytical method presented above has been validated and applied to a population of boars and gilts in order to suggest urinary 19-noretiocholanolone screening and confirmatory biomarker thresholds for the detection of nandrolone abuse. The establishment of these thresholds is significant since there is currently no internationally accepted method for detecting nandrolone abuse in this species.

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