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Project identification

1. Defra Project code: VM02143
2. Project title: Approaches to the Control of Boldenone Abuse in Cattle: (3) Metabolism studies
3. Contractor organisation(s): HFL
4. Total Defra project costs (agreed fixed price): £ 161,800
5. Project: start date ..... 01 January 2004
   end date ..... 30 April 2006

6. It is Defra's intention to publish this form.
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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

The effective enforcement of the current EU ban on the use of growth promoting agents in livestock requires suitable methodologies for the detection of key analytes in available matrices. In veterinary drug residue analysis there has been considerable interest in boldenone over the past few years, not least due to its possible endogenous nature.[1] Studies have historically concentrated on 17α- and 17β-boldenone, with more recent studies (Le Bizec et al and Sterk et al ; Euro Res V, 2004) identifying further phase I metabolites and others exploring the potential origins of boldenone and related analytes in untreated animals.[2]
There have been a number of studies investigating the presence of boldenone in urine samples contaminated with faecal material during sample collection. Such samples have been shown to be positive for the presence of 17α-boldenone in the unconjugated state. The outcome from these studies has been the establishment of the current EU regulations defining the presence of conjugated 17β-boldenone at any level as constituting a positive finding. However, these studies have been primarily related to veal calf production as representative of continental European farming practices. U.K. meat consumption is generally from animals of greater maturity with significantly less veal production. The overall aim of this study was intended to provide additional information on the metabolism and excretion of boldenone and its metabolites following administration of boldenone, boldenone esters (these being internationally available as veterinary preparations outside the EU - typically as the undecylenate ester) and prohormones (found in various 'nutritional supplements' available via numerous internet websites)

The objectives of the study were:-

Carry out administrations of boldenone, a boldenone prohormone (1,4-androstadiene-3,17-dione) and a boldenone ester.
Identify key urinary metabolites and establish metabolic profiles following the different routes of administration.
Survey a population of 100 blank urines to detect the possible presence and quantity of boldenone and metabolites.

Supplementary objectives were to develop and validate screening and confirmatory methodologies.

Following the administration of the various boldenone preparations, samples of plasma and urine were collected and analysed using solid phase extraction and GCMS analytical techniques.
Metabolites were identified in both matrices and the array of different compounds detected was broadly similar following the various routes of administration.
The metabolites have been profiled in urine and plasma against time for the different routes of administration.
Following oral administration, as expected, clearance and excretion of metabolites was fairly rapid from plasma and urine; being 2-3 and 3-4 days respectively.
Following the intramuscular administration of the undecylenate ester of boldenone, metabolites were still detectable 7-8 weeks after dosing.
Of the various metabolites detected one, tentatively identified as 6β-hydroxy-17α-boldenone, would appear to be the most promising for the detection of abuse of boldenone in either plasma or urine. No reference standard is currently available to confirm the identity of this metabolite but its mass spectra and GC chromatographic properties are consistent with it being this compound.
It was possible to confirm the presence of this metabolite in the conjugated fraction of urine samples for the duration of the sample collection period following the administration of the undecylenate ester (i.e. some 7-8 weeks post dose)
Due to the reference material for 6β-hydroxy-17α-boldenone being unavailable, methods capable of being validated to SANCO requirements could not be developed for this analyte. The method developed for screening and confirming boldenone abuse were therefore based upon the detection of 17α- and 17β-boldenone by LC-MS/MS, this has been reported separately as part of VM02143. The screening / confirmatory methods were tested to see if they would be applicable should a standard reference material become available for 6β-hydroxy-17α-boldenone, the results from this aspect of the study were inconclusive. The LC-MS/MS methodology was applied to 100 blank samples, no confirmable evidence of 17α- or 17β-boldenone was observed.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

SUMMARY

The objectives of the study were:-

To carry out administrations of 17β-boldenone, a boldenone prohormone (1,4-androstadiene-3,17-dione) and a 17β-boldenone ester.

To identify key urinary metabolites and establish metabolic profiles following the different routes of administration.

To survey a population of 100 blank urines to detect the possible presence and quantity of boldenone and metabolites.

Supplementary objectives were to develop and validate screening and confirmatory methodologies.

Following the administration of the various boldenone preparations, samples of plasma and urine were collected and analysed using solid phase extraction and GC-MS analytical techniques. Metabolites were identified in both matrices, the array of different compounds detected was broadly similar following the various routes of administration. The metabolites have been profiled in urine and plasma against time for the different routes of administration. Following oral administration of a supplement, as expected, clearance and excretion of metabolites was fairly rapid from plasma and urine; being 2-3 and 3-4 days respectively. Following the intramuscular administration of the undecylenate ester of boldenone, metabolites were still detectable 7-8 weeks after dosing. Of the various metabolites detected one, tentatively identified as 6β-hydroxy-17α-boldenone, would appear to be the most promising for the detection of abuse of boldenone in either plasma or urine. A second potentially useful metabolite the stereochemistry of which could not be established even tentatively was 5z-androst-1-ene-3z-ol-17-one No reference standard is currently available to confirm the identity of either of these metabolites. The 6β-hydroxy-17α-boldenone metabolite could be detected in the conjugated fraction of urine samples for the duration of the sample collection period following the administration of the undecylenate ester (i.e. some 7-8 weeks post dose). Due to the reference material for 6β-hydroxy-17α-boldenone being unavailable, methods capable of being validated to SANCO requirements could not be developed for this analyte. The method developed for screening and confirming boldenone abuse were therefore based upon the detection of 17α- and 17β-boldenone by LC-MS/MS, this has been reported separately as part of VM02143. The results of this research project confirmed the appropriateness of using 17α-boldenone in urine to screen for the potential abuse of boldenone. No conclusions regarding the validity of an effective reporting limit of 2ngml^{-1} for this analyte could be drawn from the results. The screening / confirmatory methods were tested to see if they would be applicable should a standard reference material become available for 6β-hydroxy-17α-boldenone, the results from this aspect of the study were inconclusive. The LC-MS/MS methodology was applied to 100 blank samples, no confirmable evidence of 17α- or 17β-boldenone was observed.

The results from this project indicate the following as desirable advances:

Production of standard reference materials for 6β-hydroxy-17α-boldenone and/or 5z-androst-1-ene-3z-ol-17-one.

Production of a standard reference material for 17α-boldenone glucuronic acid conjugate (preferably with and without deuterium label).

Further work to establish if 17α- and 17β-boldenone are endogenous and if so, to determine normal levels for these compounds.

INTRODUCTION

The use of artificial growth promoting agents in food producing animals is banned in the European Union (directive 88/146/EEC). Boldenone (17β-boldenone or 1-dehydrotestosterone) is a potent anabolic steroid, the esters of which are known to provide a long duration of action. After intramuscular treatment of the bovine the parent steroid, along with its 17-alpha epimer (a major metabolite), 1,4-androstadiene-3,17-dione and at least one major metabolite of unknown stereochemistry are excreted in urine (Van Puymbroek *et al.,* 1998). Boldenone, despite its close similarity to testosterone, has been regarded as an exogenous steroid. However, recently it was detected in the testicles of male pigs (De Brabander *et al.,* 2004) and is reported to be present at low levels in the urine of the male horse. More worryingly both 17α- and 17β-boldenone have been found in 'blank' bovine faeces

and in a test system used to study the biodegradation of testosterone. However, these findings have been clarified with respect to degradation (as such, boldenone has not been detected in fresh faecal samples). Nevertheless, several of these findings call into question the validity of boldenone as a marker analyte for the abuse of the parent compound and its esters. In order to provide an appropriate means of controlling the abuse of boldenone in the bovine a better understanding of the nature and levels of endogenous material is required along with a more detailed knowledge of its metabolism. Steroid supplements as 'over the counter; products are now readily available and have potential for abuse in livestock. Such products also need to be considered in the development of an effective strategy to control boldenone abuse.

EXPERIMENTAL

Animal Administrations

A sequential administration involving 2 beef cattle and 3 preparations of boldenone was carried out. Boldenone was given as an intra-muscular bolus administration, followed by an oral administration of the supplement 1,4-androstadiene-3,17-dione and finally an intra-muscular administration of a boldenone ester preparation (17β-boldenone undecylenate).
The cattle were allowed a 7 day acclimatisation period during which blank urine and blood samples (x2) were collected.

Boldenone bolus administration
400 mg 17β-boldenone was administered as a bolus IM dose in its natural unesterified state. Blood and urine samples were collected from the animals over a period of 14 days. An additional sample was collected just prior to the supplement administration.

Supplement /prohormone administration
After 14 days the cattle were administered 500 mg of an oral supplement (1,4-androstadiene-3,17-dione) and as before, a series of blood and urine samples was collected for a period of 10 days. As with the 17β-boldenone administration, an additional sample was collected prior to the 17β-boldenone ester administration.

Boldenone undecylenate ester administration
The cattle were then administered 700 mg of an intramuscular preparation of 17β-boldenone undecylenate ester. Blood and urine was collected daily for the first 7 days, and then at 2 day intervals until day 56 post administration.
Faecal samples were also collected prior to administration and 48 hr post administration for all 3 administrations and again on day 5 post administration for the boldenone ester preparation, the analysis of these samples was not included as part of this research project. The study was terminated 56 days after the administration of the boldenone ester and samples of bile, muscle and the injection sites were collected following slaughter.

The administrations were carried out by the VLA (Veterinary Laboratories Agency) Weybridge, Surrey.

Extraction Methods

Urine sample extraction

Post administration urine samples (5ml) were spiked with internal marker ($^2H_3$-testosterone). The aliquots were then extracted using a manual solid phase extraction procedure, which enabled separate free and conjugated fractions to be prepared. To accomplish this, the free steroid fraction was eluted from the cartridge using diethyl ether and the conjuqated steroid fraction was eluted from the cartridge using methanol. The conjugated fraction eluates were evaporated to dryness, reconstituted in phosphate buffer containing β-glucuronidase from *E.coli,* the extract was then incubated in order to hydrolyse glucuronic acid conjugates. After incubation the extract was basified using aqueous sodium hydroxide and a liquid-liquid extraction was performed using diethyl ether. The resultant extract was evaporated to dryness and reconstituted in organic solvents. A further solid phase silica clean-up procedure was carried out and enol-TMS derivatives were formed prior to GC-MS analysis.

Plasma sample extraction

Post administration plasma samples (3ml) were extracted using the procedure outlined for urine above except the internal marker used was $^2H_3$-17β-boldenone (50ng), the silica clean-up extraction stage was not performed on these samples and analysis was only carried out using GC-MS. Semi-quantitation was attempted for 17α- and 17β-boldenone, 6β-hydroxyboldenone and 1,4-androstadiene-3,17-dione. Where no reference standards were available for other relevant metabolites, peak area ratios were calculated. Steroid stripped bovine plasma was prepared and used for calibration samples.

GC-MS Analysis

All GC-MS analyses were carried out in the positive ion electron impact (EI) mode. Dependant upon the requirement for the analyses carried out a variety of instruments and columns were used:

For full scan GC-MS analysis to identify the key metabolites in the post administration urine samples analysis was performed using a Finnigan Voyager bench top quadrupole instrument and an SGE BPX5 (low polarity) column.

GC-MS/MS analysis for further elucidation of urinary metabolites was performed using a Finnigan GCQ bench top quadrupole ion trap instrument and a SGE BPX50 (medium polarity) column.

For semi-quantitative GC-MS analysis of urine samples in order to produce excretion profiles for the metabolites identified during the qualitative evaluation, selected ion monitoring (SIM) experiments were carried out using a Finnigan Voyager bench top quadrupole instrument and an SGE BPX5 (low polarity) column.

GC-MS/MS analysis of plasma extracts to establish circulating steroid profile following drug administration were carried out using a Varian 1200 bench top triple quadrupole instrument and an SGE BPX5 column

Quantitative LC-MS/MS Analysis of urine samples.

17α and 17β-boldenone levels in the boldenone undecylenate ester post administration urine samples were quantified using LC-MS/MS. Steroid stripped bovine urine was prepared and used for calibration samples. LC-MS/MS analysis was also performed on a batch of 100 blank urines to establish the possible presence of 17α and 17β-boldenone in 'normal' samples. Samples were analysed using positive electrospray ionisation in the MRM mode on a Waters Micromass Quattro Premier bench top triple quadrupole instrument and a Waters 1525µ binary gradient HPLC. Sample injection volume was 20ul and chromatography was performed on a Phenomenex Luna C18(2), 150 x 2.0 mm, 5 µm at ambient temperature using mobile phases ammonium formate, 25mM, pH4.5 (A) and methanol (B). Gradient elution was performed with initial composition of 60% B held for 1min, rising to 90%B at 15mins held for 1 min, returning to initial start conditions and re-equilibrated for a further 6mins. MRM transitions were as follows.

MRM of 13 mass pairs, Time 0.5 to 15 min, ES+

Channel	Parent (Da)	Daughter (Da)	Dwell (secs)	Cone (V)	Collision (eV)
1	271.20	199.20	0.2	20	18
2	271.20	253.20	0.2	20	15
3	275.20	109.03	0.2	20	16
4	275.20	145.07	0.2	20	16
5	278.30	145.09	0.2	30	22
6	278.30	148.10	0.2	30	20
7	287.25	121.00	0.2	20	22
8	287.25	135.05	0.2	20	14
9	290.10	121.00	0.2	35	25
10	290.10	137.90	0.2	35	15
11	303.10	97.00	0.2	20	15
12	303.10	109.00	0.2	20	18
13	306.20	108.90	0.2	40	20

RESULTS AND DISCUSSION

GC-MS analysis of post administration urine samples

The major urinary metabolites identified in the glucuronic acid conjugated fraction following administration of 17β-boldenone (where authenticated reference standards were available) were, 17β-boldenone, 17α-boldenone, 6β-hydroxy-boldenone and 5β-androst-1-ene-17β-ol-3-one. A number of other metabolites were also detected. These have been assigned the following structures, 6β-hydroxy-17α-boldenone, 5β-androst-1-ene-17α-ol-3-one, and 5z-androst-1-ene-3z-ol-17-one. However, in the absence of authenticated reference standards the identification of these must be considered tentative. The levels of 17β-boldenone detected were significantly lower than that of the major metabolites. The proposed 6β-hydroxy-17α-boldenone metabolite shows a mass spectrum (Figure 1) with identical mass ions to that obtained from an authentic reference standard of 6β-hydroxy-17β-boldenone, (Figure 2). The earlier retention time is characteristic of 17α-epimers of trimethylsilylated steroids analysed using the GC conditions described. This metabolite was identified as a strong candidate for a marker analyte for the abuse of boldenone in the bovine.

A further candidate analyte marker of boldenone abuse was detected. The mass spectrum of this analyte (Figure 3) was detected in the glucuronic acid fraction of post administration urine samples and is proposed as the metabolite 5z-androst-1-ene-3z-ol-17-one, with unknown stereochemistry. This metabolite has been observed in human urine following the oral administration of boldenone[3]. It represents a metabolite that has undergone three phase I transitions (including reduction at carbon C5) followed by phase II conjugation and as such, is less likely to be derived from bacterial activity in urine, making it a potentially useful, additional, marker metabolite for boldenone administration.

Following oral administration of 1,4-androstadienedione no significant difference in the identity of the metabolites detected was noted. In the free steroid fraction 17α-boldenone, 5β-androst-1-ene-17α-ol-3-one, 6β-hydroxy-17β-boldenone and 5β-androst-1-ene-17β-ol-3-one were detected (where reference steroids are not available identifications are tentative as noted previously). The same metabolites were detected in more significant quantities in the glucuronic acid conjugate fraction for the first 24 hours post dose. A further metabolite, 6β-hydroxy-17α-boldenone was also detected in the glucuronide fraction. 17α-boldenone levels in the conjugated fraction peaked at approximately 700ngml^{-1} after a few hours. Following the oral administration of the supplement, metabolites were detectable in urine in the glucuronic acid fraction for 3 to 4 days post dose as can be seen in Figure 4.

Figure 1– Mass spectrum of a metabolite assigned the structure 6β-hydroxy-17α-boldenone – tris-TMS (RT=16.13) obtained from a post administration urine extract

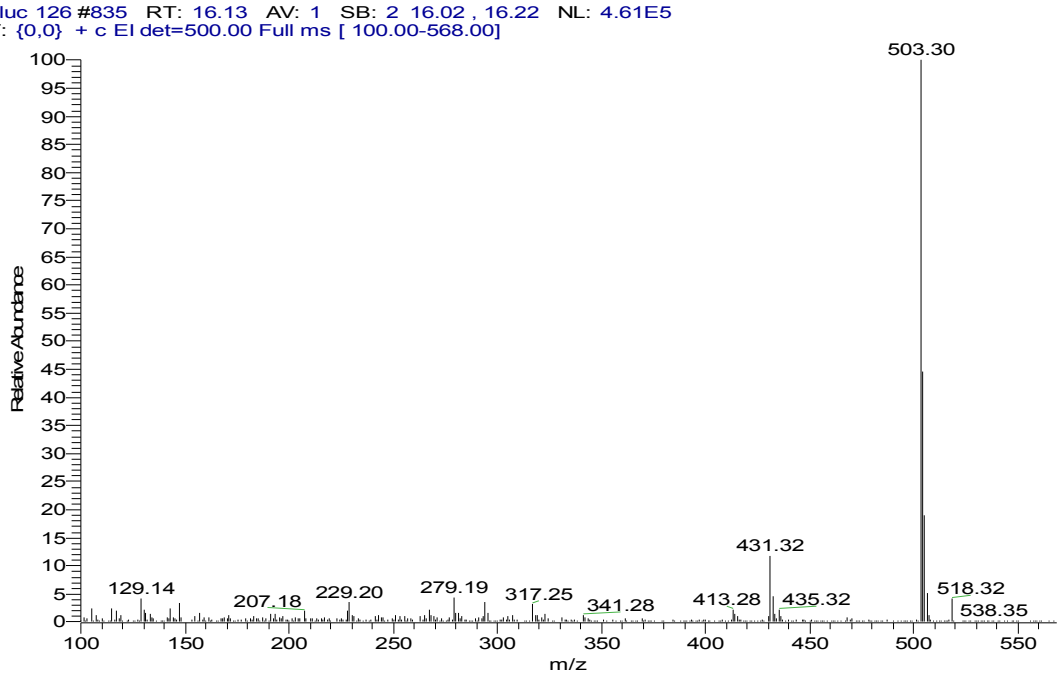


Fig 2 – Mass spectrum of 6β-hydroxy-17β-boldenone – tris-TMS (RT=16.98) Reference standard

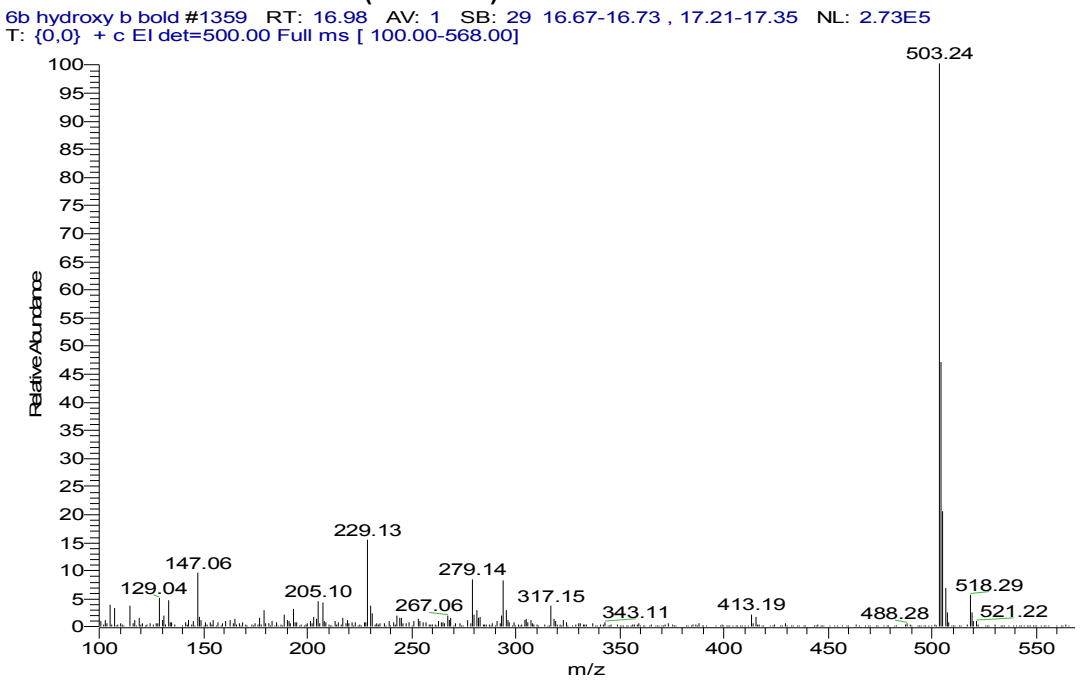


Fig 3 - Mass spectrum of a metabolite assigned the structure 5z-androst-1-ene-3z-ol-17-one – *bis*-TMS (RT = 13.46) obtained from a post administration urine extract

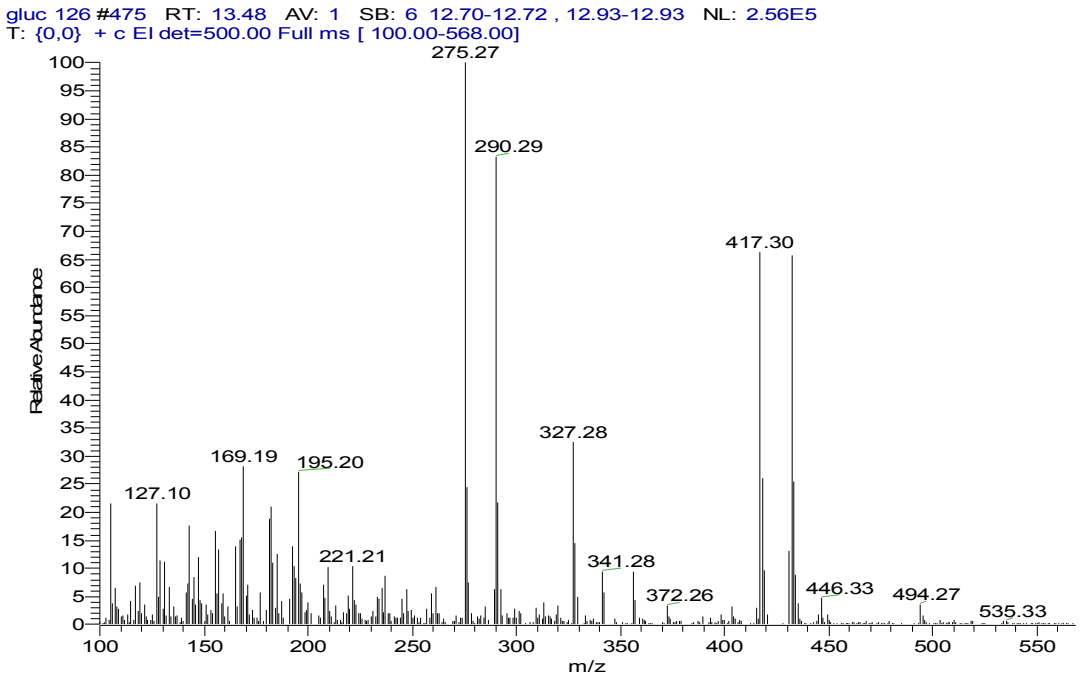
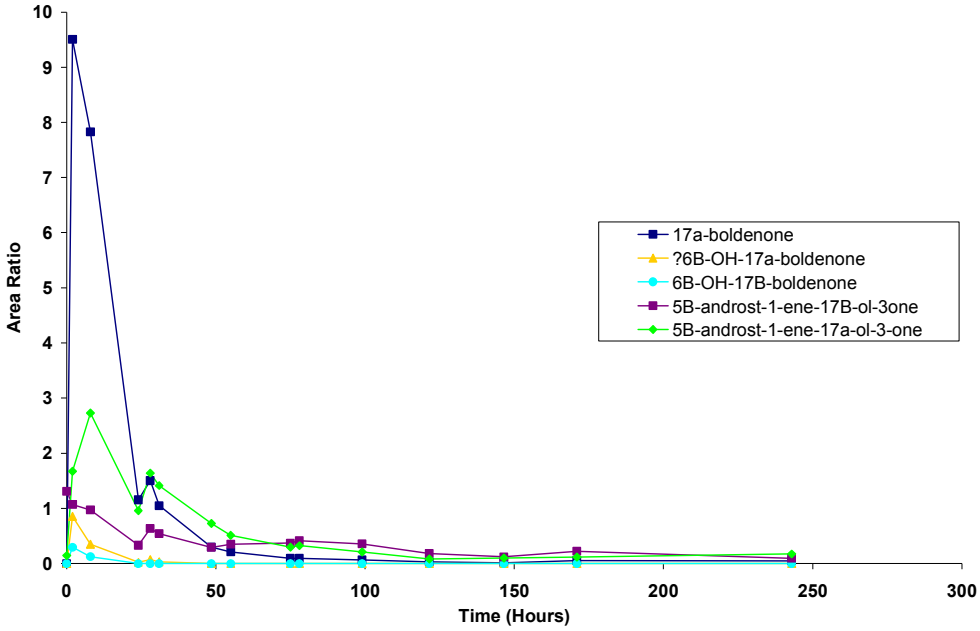
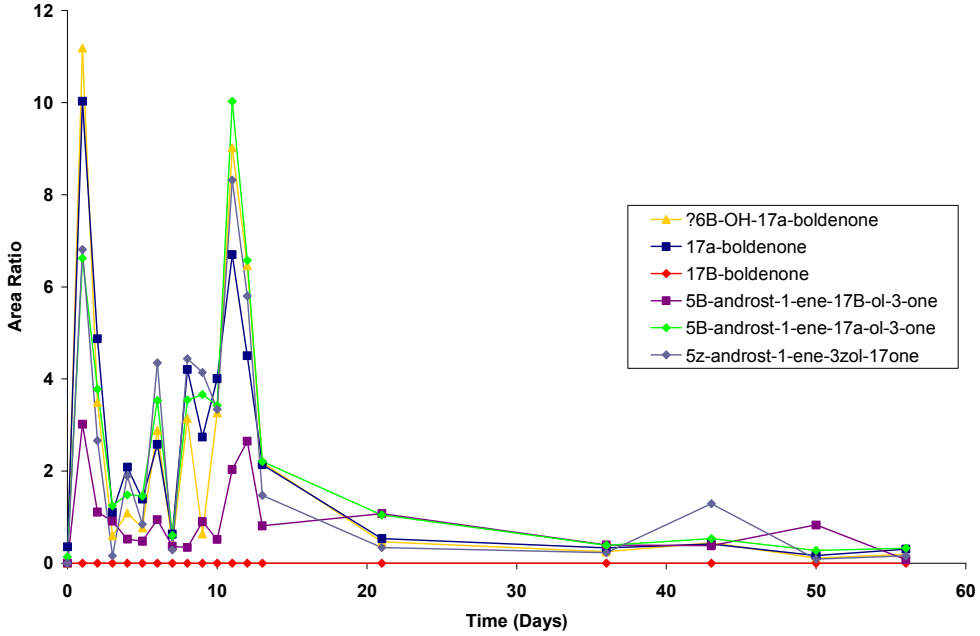


Figure 4: Urinary profile of boldenone metabolites in the glucuronic acid conjugate fraction following oral administration of 1,4-androstadiene-3,17-dione.



Following the intra-muscular administration of the 17β-boldenone undecylenate ester, 17α-boldenone, 5β-androst-1-ene-17α-ol-3-one, 6β-hydroxy-17α-boldenone, 5β-androst-1-ene-17β-ol-3-one and 5z-androst-1-ene-3z-ol-17-one were all detected in the urinary glucuronic acid conjugate fraction. Following administration of the ester, metabolites were detectable in urine in the glucuronic acid fraction for the duration of the sample collection period as can be seen in Figure 5.

Figure 5: Urinary profile of boldenone metabolites in the glucuronic acid conjugate fraction following intra-muscular administration of 17β-boldenone undecylenate.



GC-MS analysis of post administration plasma samples

The bolus administration served as an analytical tool to provide information on the metabolites likely to arise following the other routes of administration, but in more readily detectable quantities. Following the intramuscular administration of a 17β-boldenone bolus, 17β-boldenone, 6β-hydroxy-17β-boldenone, 17α-boldenone and 6β-hydroxy-17α-boldenone were all observed in both the free and glucuronic acid conjugate fractions. The 17α-epimers were present in significantly greater quantities in the conjugated fraction than the free fraction and vice-versa for the 17β- epimers.

Following the oral administration of 1,4-androstadienedione, 17α-boldenone and 6β-hydroxy-17α-boldenone were detected in the plasma free fraction and in more significant quantities (approximately 12ngml^{-1}) in the glucuronic acid conjugate group fraction for the first 4-5 hours post dose. 17β-Boldenone and 6β-hydroxy-17β-boldenone were detected in both fractions, though only briefly at very low levels, indicating extensive 17α-epimerisation occurs during phase I metabolism. This potentially brings into question the efficacy of the prohormone, if its suggested benefits are to be mainly derived from the introduction of 17β-boldenone into the general circulation. The administration of the supplement was detectable for at least 24hrs in plasma and possibly up to 48 hours using the two aforementioned metabolites as markers (Figure 6).

Following intra-muscular administration of 17β-boldenone undecylenate ester, 17β-boldenone was detected in the free fraction for the duration of the 56 day sample collection period (Figure 7), peak levels were approximately 1ngml^{-1}. No other analytes were observed at significant levels. Three major analytes were detected in the glucuronic acid conjugate fraction, namely 17α-boldenone (maximum level approximately 300pgml^{-1}), 17β-boldenone (maximum level approximately 100pgml^{-1}), and 6β-hydroxy-17α-boldenone (no standard available, maximum level estimated as 150pgml^{-1}), (Figure 8). These levels are below the lowest point of the calibration curve and approaching the capability of the analytical method which in some way explains the variability of the profile. Despite this, good qualitative data for the presence of these metabolites for up to 4-5 weeks post dose with possible increased duration of detection using the 6β-hydroxy-17α-boldenone metabolite was obtained.

Figure 6: Plasma profile of boldenone metabolites in the glucuronic acid conjugate fraction following oral administration of 1,4-androstadiene-3,17-dione

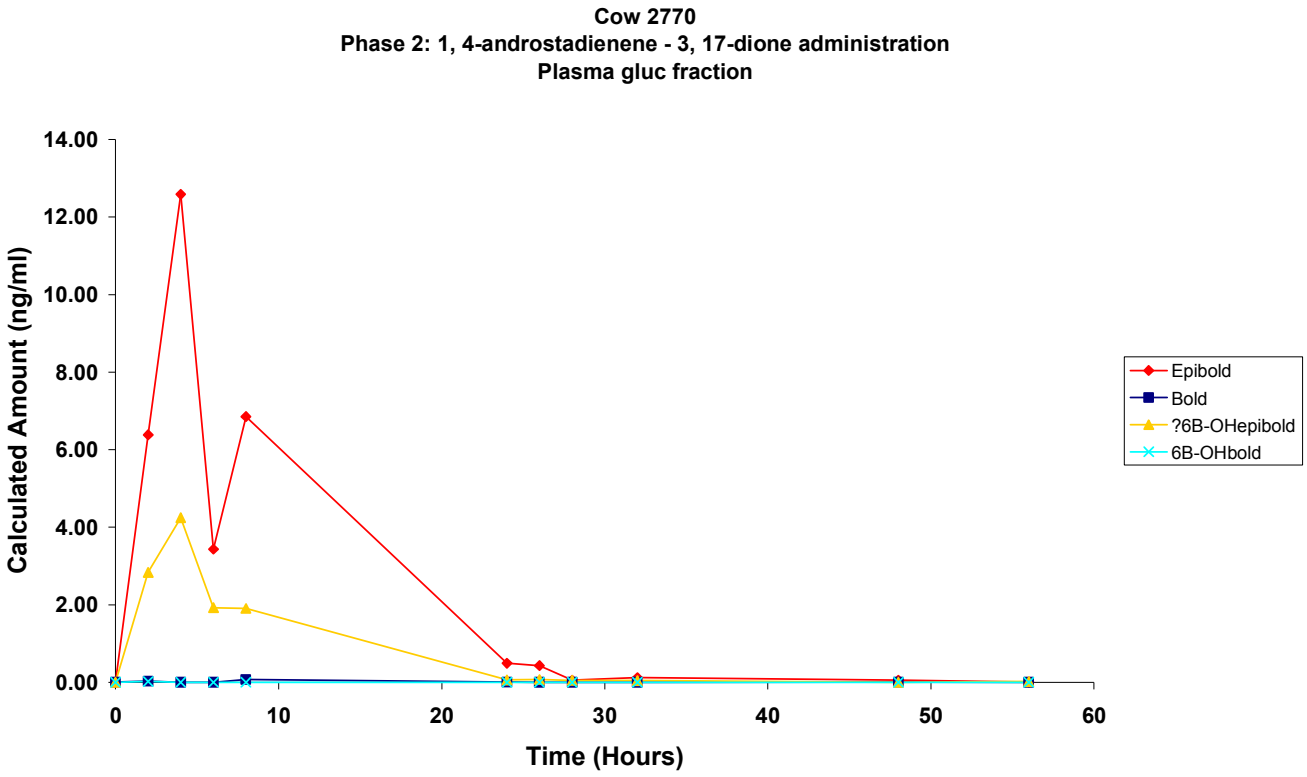


Figure 7: Plasma profile of 17β-boldenone in the free fraction following intra-muscular administration of 17β-boldenone undecylenate

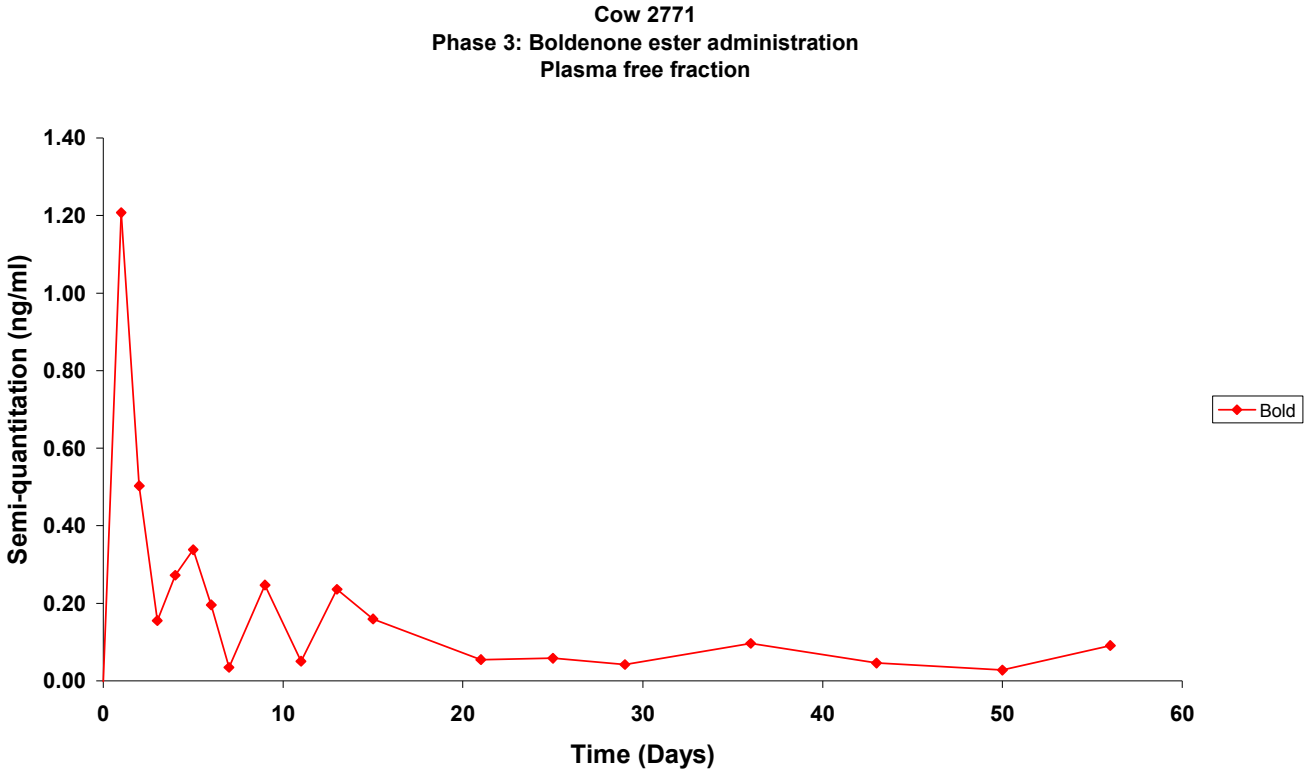
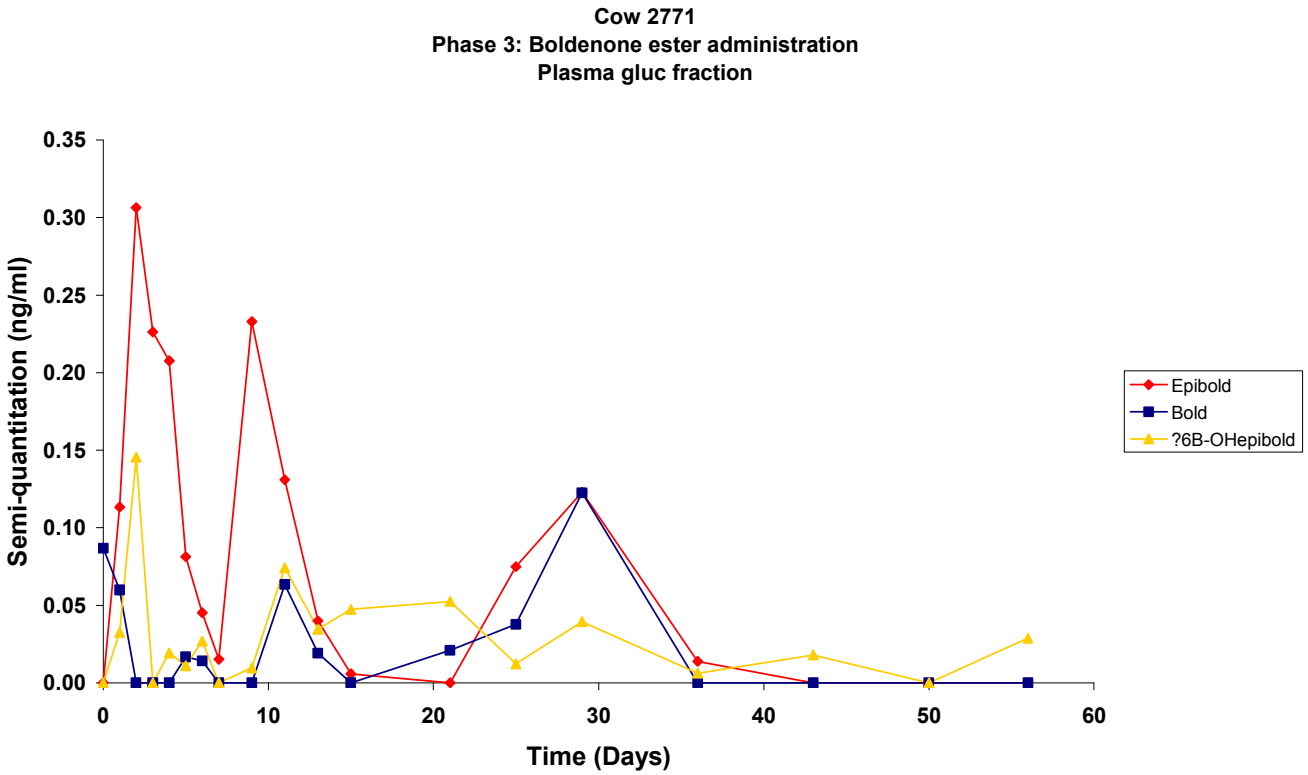


Figure 8: Plasma profile of boldenone metabolites in the glucuronic acid conjugate fraction following intra-muscular administration of 17β-boldenone undecylenate



Quantitative LC-MS/MS analysis of urine samples following intra-muscular administration of 17β-boldenone undecylenate.

An objective of the project was to analyse at least one hundred nominally 'blank' samples for the presence of marker metabolites of boldenone abuse. A further additional objective was to develop screening and confirmatory assays for boldenone abuse. While this project identified at least two potential markers, in addition to the proposed EU approach using the detection of 17α and 17β-boldenone glucuronic acid conjugates, reference materials for these alternative markers were unavailable. Free and glucuronic acid conjugate fractions of urine samples obtained following intra-muscular administration of 17β-boldenone undecylenate were therefore analysed using LC-MS/MS. The glucuronic acid conjugate fraction of one hundred 'blank' samples was also analysed.

As expected, the levels of both 17α and 17β-boldenone in the free fraction of the post administration urine samples were very low, of the order of 10ngml^{-1} maximum for 17α-boldenone and sub nanogram for 17β-boldenone. In the glucuronic acid fraction the levels of 17α-boldenone detected were elevated significantly, up to approximately 200ngml^{-1}, and allowed detection of administration for the duration of the study, 56 days, 17β-boldenone was only detected in the low ngml^{-1} range. In none of the one hundred 'blank' urine samples could the presence of either 17α- or 17β-boldenone be confirmed.

Assuming the data from the intra muscular administration of the ester is representative of the profile expected of a larger population of administration studies it raises some interesting issues. Clearly, 17α-boldenone glucuronic acid conjugate is a major metabolite of 17β-boldenone and it can be used to detect possible abuse of 17β-boldenone, its esters and precursors. However, this approach is clearly affected by the observation that free 17α-boldenone can be produced by bacterial action upon faecal contaminated urine. The fact that no confirmable findings of 17α-boldenone were observed in the 'blank ' urine samples analysed supports the value of 17α-boldenone glucuronic acid conjugate as a marker of abuse. However, the question remains 'is 17α-boldenone an endogenous substance and if so at what level? Is the arbitrary limit of 2ngml^{-1} appropriate? Current guidelines also suggest that detection of 17β-boldenone at any level is indicative of abuse. The data presented suggests that a highly sensitive methodology is required to detect the trace levels of 17β-boldenone present following administration, particularly of the long acting ester formulation. Application of sufficiently sensitive assays may yet indicate that 17β-boldenone is an endogenous steroid, as has proved to be the case in the male equine. Such a finding in the bovine would significantly impact the current approaches to the control of boldenone abuse as a growth promoting agent.

CONCLUSIONS

Following the administration to the bovine of a 17β-boldenone undecylenate ester or the supposed precursor of 17β-boldenone, 1,4-androstadiene-3,17-dione, 17β-boldenone glucuronic acid conjugate does not appear to be excreted in significant quantities. Current EU guidelines used by laboratories carrying out meat residue analysis to support statutary testing programmes suggest that detection of 17β-boldenone glucuronic acid conjugate at any level is indicative of the abuse of boldenone. While the data obtained in this report does not contradict that proposition it is clear that highly sensitive methods would be required to detect the abuse of boldenone using this marker. Given that evidence exists that 17β-boldenone is an endogenous steroid in the male equine and that other steroids related to testosterone, for example nandrolone, once thought to be entirely exogenious have been found at trace levels in a number of species, a strong possibility exist that given sufficiently sensitive assays 17β-boldenone may yet be found to be an endogenous steroid in the bovine.

The data from this project suggests that any urine sample containing small quantities of 17β-boldenone glucuronic acid conjugate as a result of the abuse of boldenone should also contain more significant quantities of and hence more readily detectable alternative phase II conjugated metabolites. Current guidelines also suggest that the presence of 17α-boldenone glucuronic acid conjugate at a level above 2ngml^{-1} in bovine urine represents a suspicious finding. The validity of what is in effect an MRPL of 2ngml^{-1} for this analyte cannot be established from the data presented here. However, it is clear that levels of 17α-boldenone glucuronic acid conjugate significantly higher than 2ngml^{-1} arise from the administration of 17β-boldenone undecylenate ester or 1,4-androstadiene-3,17-dione. Given the availability of reference materials for 17α-boldenone, the potential of this analyte to screen for abuse of boldenone is supported by the results of this project. It is considered likely that the availability of a reference standard of 17α-boldenone glucuronic acid conjugate (preferably also as a deuterated analogue) would significantly enhance the ability of laboratories to reliably detect and quantify this analyte.

While 17α-boldenone is a useful marker of boldenone abuse in the bovine at least two further markers were identified in this project. The most prevalent of these is the metabolite tentatively assigned the structure 6β-hydroxy-17α-boldenone. There is no evidence in the literature that this metabolite could arise due to faecal

contamination, also it was detected at significant levels in the glucuronic acid conjugate fraction of post-administration urine samples regardless of the route of administration and some evidence for its presence in the circulating medium was found. Clearly, if a reference material was available, 6β-hydroxy-17α-boldenone would prove to be a highly desirable marker of the use or abuse of bodenone.

A second attractive alternative marker is 5z-androst-1-ene-3z-ol-17-one, also detected in the glucuronic acid conjugate fraction. A significant reason why this could represent a useful marker metabolite for the administration of boldenone is that it has undergone three phase I transformations followed by phase II conjugation, reducing the likelihood of it being derived from faecal contamination or bacterial degradation. Certain fungi have been shown to produce both phase I and phase II metabolites of xenobiotics[4], although these are 'biologically available' for consumption, it is considered unlikely that they would survive the low pH of stomach acids.

Analysis of plasma samples using GC-MS/MS techniques indicated that detection of circulating boldenone metabolites is possible in this medium. Plasma has many attractions as a analytical matrix, not least its homogeneity, ease of collection and low probability of contamination. Also, compared to urine, for small molecules, plasma contains relatively low levels of potential interferences. The obvious counter to this argument is that very sensitive methods are required. Until recently analysis of steroids in plasma at realistic levels was beyond the instrumental capability and analytical skills of most residue testing laboratories. Enhancements in instrumentation and related improvements to, for example, extraction procedures have significantly impacted this situation.

On current evidence it would appear that efficient removal of the free steroid fraction prior to enzyme hydrolysis provides a relatively straight forward approach to detection of conjugated boldenone metabolites in bovine urine and this can provide convincing evidence of boldenone abuse. The use of alternative analytes highlighted in this research could provide greater confidence and possibly extended detection times. For these advantages to be obtained there is a need to produce authentic standard reference materials for these analytes. In the absence of these standards becoming available additional research to establish if 17α or 17β-boldenone are in fact endogenous steroids and to determine their concentration in a large population would be desirable. Plasma represents a potentially suitable matrix for the detection of the administration of boldenone but this would require highly sensitive methods to support this.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

[1] Arts et al; Euro Res III, **1996** p212-217

[2] Brabander et al; Presence and metabolism of the anabolic steroid boldenone in various animal species: a review; Food Additives and Contaminants, Vol. **21**, No.6, **2004**, pp 515-525

[3] Schanzer et al; Metabolism of Boldenone in man: Biological Mass Spectrometry, vol.21, 3-16 (1992)

[4] Zhang et al., FEMS Microbiology Letters 138 (1996) 221-226