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Project identification

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

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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 objectives of the project were two-fold:

1) To develop a residue screening method for the analysis and detection of 17α- and 17β-boldenone in bovine urine at the minimum required performance limit (MRPL) of 1ngml^{-1} and an agreed action limit of 2ngml^{-1}.

2) To carry out a multi-site validation exercise consistent with the guidelines laid out in SANCO/1085/2000 Revision 6.

Objectives were largely attained in that:

An LC-MS/MS method for the qualitative and semi-quantitative screening analysis of 17α- and 17β-boldenone in bovine urine was developed based on a generic method currently being used by the residue testing laboratory of LGC Ltd. Optimisation of enzyme hydrolysis, cartridge elution and internal standard selection were carried out.

The ruggedness of the screening methodology was tested using a Youden approach. No loss of performance was observed following the deliberate alteration of the pre-selected critical factors of the procedure. Therefore, no modifications to the methodology were needed. It was shown that the use of the deuterium labelled internal marker successfully accounted for any variation in the absolute recovery of the analyte.

A standard operating procedure (SOP) for the methodology has been produced in a format consistent with the ISO75-2 guidelines.

The stability of 17α- and 17β-boldenone in standard solutions and sample extracts was established under a variety of conditions. 17α- and 17β-boldenone were shown to be stable in fortification solvent solutions for up to 20 weeks at room temperature under normal lighting conditions. 17α- and 17β-boldenone were shown to be stable in final analysis solutions for up to one week after storage at room temperature under normal lighting conditions.

The methodology was shown to be specific for 17α- and 17β-boldenone in that these compounds were distinguishable from other 17β-boldenone analogues and metabolites.

The working range of the quantitative method was established as 0.5ngml^{-1} to 4ngml^{-1}. High relative recovery (losses accounted for using deuterated internal standard), good precision and good linearity for calibration point data were obtained over this range.

The decision limit, CCα of the methodology was calculated as 0.8ngml^{-1}, 1.0ngml^{-1} and 1.0ngml^{-1} for 17α-boldenone and 0.2ngml^{-1}, 0.5ngml^{-1} and 1.0ngml^{-1} for 17β-boldenone, from results returned by the participating laboratories, DARD, HFL and LGC, respectively.

All positive identifications of 17α-boldenone or 17β-boldenone needed to satisfy criteria that the ratio of the chromatographic retention time of the analyte to that of the internal standard, ie the relative retention time (RRT), must correspond to that of the system suitability standard at a tolerance of +/- 2.5% and that the relative intensity of two characteristic transitions were within the criteria specified in the SANCO guidelines.

The results obtained from the evaluation indicate that the proposed screening methodology is adequately robust for its intended use. This conclusion is based upon the methodology demonstrating applicability, ruggedness and robustness for the analysis of 17α-boldenone and 17β-boldenone at the reporting level of 2ngml^{-1}. The methodology appears to be reaching the limits of its capability for 17α-boldenone at about 1ngml^{-1}. This is not considered a major issue as current knowledge indicates that the presence of 17β-boldenone is much more indicative of potential abuse of boldenone, its esters or precursors.

The laboratories that collaborated with HFL in the method validation were:

LGC Ltd (residue testing laboratory), Teddington, Middlesex, UK
Department of Agriculture and Rural Development (DARD), Queen's University Belfast, UK
Central Science Laboratory (CSL), Sand Hutton, York, UK

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).

INTRODUCTION

The use of artificial growth promoting agents in food producing animals is currently banned in the European Union (directive 88/146/EEC). 17β-boldenone (alternatively known as 1-dehydrotestosterone) is a potent anabolic steroid and has the potential for abuse in livestock. Therefore, an effective strategy to control potential 17β-boldenone abuse is required.

Current scientific knowledge in this field indicates that analytical screening of 17α-boldenone and 17β-boldenone in bovine urine (following enzyme hydrolysis), is appropriate to detect the abuse of 17β-boldenone, its esters and prohormones, when used as growth promoting agents in the bovine. A recently published review article

summarises research carried out in this area {Presence and metabolism of the anabolic steroid 17β-boldenone in various animal species: a review: Food Additives and Contaminants, Vol. 21, No.6 (June 2004), pp.515-525}.

The National Surveillance Programme requires an analytical strategy for the regulatory control of 17α-boldenone and 17β-boldenone in the bovine. For the purposes of the United Kingdom statutory monitoring programme, methods validated to SANCO guidelines are required. It is also desirable that methods are shown to be appropriate and robust by validation across laboratories involved in residue analysis and the statutory testing programme. The aim of this aspect of the project was to develop and validate a screening method for 17α-boldenone and 17β-boldenone with a minimum required performance limit of $1ngml^{-1}$ in bovine urine. Liquid chromatography with tandem mass spectrometry detection (LC-MS/MS), following addition of an internal marker and extraction of the analytes, was chosen as the analytical technique of choice due its frequent application in UK statutory testing facilities.

In the first instance a generic LC-MS/MS methodology used by LGC in their residue testing laboratory at Teddington (UK) for screening steroids in bovine urine was investigated and optimised for the analysis of 17α-boldenone and 17β-boldenone. This methodology is broadly similar to that employed by the Department of Agriculture and Rural Development (DARD) in their residue testing facility at Queens University Belfast (UK) for the Northern Ireland statutory program.

HFL has undertaken a full validation of the methodology and has provided DARD and LGC with blank and augmented urine samples to enable them to participate in the validation exercise. The results returned form the participating laboratories have been used to determine both inter and intra-laboratory variation.

EXPERIMENTAL

Blank matrix

Unless otherwise stated, all analyses were performed using, nominally negative, female bovine control urine, taken from animals at post mortem acquired by the Veterinary Laboratories Agency (VLA) or using negative bovine control urine, taken from 'on farm' animals involved in research studies undertaken by DARD. Prior to pooling the urine, each individual animal's urine was screened for the presence of 17α-boldenone and 17β-boldenone, and only samples found to be 'negative' were used.

Standard Preparation

Where sufficient standard was available, $1mgml^{-1}$ solutions were prepared by accurate weighing and dissolution in methanol. For $^{2}H_{3}$-17β-boldenone and 17α-boldenone only limited amounts of reference material were available and accurate weighing could not be achieved. For these analytes the weight of analyte claimed by the supplier was assumed to be correct and appropriate dissolution volumes used. Further dilutions were made to produce working solutions at a concentration of $100ngml^{-1}$.

System Suitability Standard

A system suitability standard containing all relevant analytes of interest was prepared by taking 50µl of each working solution, evaporating the solvent and reconstituting in 350µl of 50:50 water:methanol. This standard was analysed prior to each analytical run to check instrument acceptance criteria were met, ie retention times, elution order and the signal-to-noise ratio of all analytes.

Method

The methodology currently employed by the residue testing laboratory at LGC for the generic detection of steroids in bovine urine was assessed and modified where appropriate. For the purposes of SANCO validation the methodology is required as an SOP in a format consistent with the ISO75-2 guidelines. An SOP for the method can be found in Appendix I.

Ruggedness Testing

A ruggedness test based on the approach described by Youden and referenced in the SANCO/1805/2000 revision 6 guidelines (pages 30-32) was carried out. This is a fractional factorial design approach. Therefore, interactions between different factors cannot be detected. This type of study deliberately introduces minor

variations into the method. The consequences of these variations upon the detection and quantification of 17α-boldenone and 17β-boldenone were observed and determined. The Youden approach is designed not to study one alteration at a time, but to introduce several variations at once. It results in 128 different outcome combinations.

Ruggedness was tested using bovine urine augmented at a level (4ngml^{-1} 17α-boldenone and 17β-boldenone), which was regarded to be high enough to reliably detect any major differences in the response ratios for each analyte. Eight experiments were designed which tested seven different factors. The factors were investigated in duplicate thereby introducing several variations at one time. Raw data were analysed and treated by calculating the ratio of the response of the MRM transition 290>121 for $^{2}H_{3}$-17β-boldeonone (internal marker) to that of the response of the 287>121 transition for 17α-boldenone and 17β-boldenone. The average values obtained for each minor variation (factor) were compared to each other to establish if a given factor had any significant influence or effect upon the results obtained. Possible factors that could influence the results were identified in pre-investigative studies. The deviations of these factors usually encountered among and between laboratories were discussed and a level agreed. Full details of the ruggedness testing carried out and the results obtained can be found in a separate report submitted to DEFRA.

Validation

A validation plan identifying the role and requirements for each collaborating partner was produced and agreed prior to commencing validation. This plan included details of the methodology, sample preparation and reporting. The relevant part relating to the approach to validation has been abstracted and is presented in Appendix 2.

RESULTS AND DISCUSSION

Method Development

The mass spectra of all analytes showed an intense protonated molecular ion as the major diagnostic ion. Under some conditions a significant sodiated ion was also observed; control of the production of this ion is desirable to ensure maximum sensitivity and issues of variability between 17α- and 17β-boldenone. The sample treatment, extraction and analysis used was based upon that used by LGC for their generic steroid screening; this, in turn, was derived from the methodology developed and published by DARD/QUB. In essence the method consists of the enzyme hydrolysis of the urine sample to produce free steroid followed by initial extraction on a C18 solid phase extraction cartridge. The somewhat complex C18 extract is then cleaned using an NH_2 cartridge before analysis using a triple quadrupole, atmospheric pressure ionisation (API) mass spectrometer operated in electrospray, positive ion, mode (ESI+). A $^{2}H_{3}$-17β-boldenone labelled analogue was used as an internal marker and to correct for any variability of extraction or instrument response. For 17α- and 17β-boldenone the MRM transition m/z 287 > m/z 121 was used as the quantifier ion, with m/z 287 > m/z 135 used as the qualifier ion; the internal marker was detected using the transition m/z 290 > m/z 121. In practice, the methodology would be used to screen for multiple analytes in a single run, for this reason validation was undertaken using a mass spectrometer methodology which included a total of 13 MS/MS transitions.

Assay Optimisation

The optimum enzyme hydrolysis conditions were determined following a series of experiments. The amount of free 17α-boldenone and 17β-boldenone recovered after the hydrolysis of an incurred sample did not change significantly when the reaction time or the volume of enzyme was increased. Based upon the results it was evident that using either 60µl of enzyme, at 37^{o}C overnight, 40µl of enzyme at 55^{o}C for three hours or 50 µl helix pomatia at 37^{o}C for 1 hour achieved complete hydrolysis.

The method to evaporate the eluent from the NH_2 cartridge was optimised. Comparison was made between the use of a heating block with nitrogen flow and a Turbovap® system. The use of a heating block proved more rapid and analysis showed no significant differences between data from the two methods.

The optimum volume and solvent composition used to reconstitute the extracts for the analysis was investigated. The use of 200µl of 60:40 Methanol:buffer (LC starting conditions) gave a slight improvement in sensitivity compared to the use of 350µl of 50:50 Methanol:Water. However, the improvement was minimal and so, as the latter is used by LGC in their generic steroid screen, it was decided to validate using this solvent system.

Ruggedness Testing

Ruggedness testing was carried out using a Youden model. Factors investigated with respect to use of the C18 cartridge included the flow rate of sample application, speed of wash application, wash solvent composition, flow rate of elution, cartridge drying time and elution solvent composition; the factor investigated with respect to use of the NH_2 cartridge was the flow rate of elution. No significant difference in recovery of 17α-boldenone or 17β-boldenone was observed for any of the minor changes introduced into the method. From the ruggedness testing results it was evident that there were no critical factors that affected the ruggedness of the methodology. The use of a deuterium labelled internal marker successfully accounted for any variation in the absolute recovery of the analytes.

Stability of 17α-boldenone and 17β-boldenone

The stability of 17α-boldenone and 17β-boldenone in solution and following extraction was investigated under various conditions. The analyte was considered stable under the given storage conditions if the back calculated concentration exceeded 85% of the nominal value. Both 17β-boldenone and 17α-boldenone proved to be stable for a minimum of 20 weeks under all storage conditions in fortification solutions.

The stability of 17α-boldenone and 17β-boldenone in the final analysis solvent was investigated under the same conditions. Both were found to be stable for a minimum of one week.

Specificity

SANCO guidelines for validation require that the method specificity should be demonstrated by a number of means including the analysis of 'blank' samples to detect the presence of possible interferences. Where the analyte is an endogenous substance or, as is the case for 17α-boldenone, may arise as an artefact it can prove difficult to assess assay specificity as the detection of a peak close to the retention time of the analyte may represent an interferance or the correct detection of endogenous material. The approach taken to assessing specificity of the method was ultimately agreed with the collaborating laboratories as the most pragmatic approach available.

The specificity of the method was tested by the analysis of twenty 'nominally negative' blank bovine urine samples each extracted and analysed for the presence of interfering peaks in the MRM transition m/z 287>121 by all three laboratories. For one sample all three laboratories reported significant amounts of 17α-boldenone, 5.9ngml^{-1}, 1.8ngml^{-1} and 2.8ngml^{-1} (DARD, HFL and LGC, respectively). Small peaks were observed at the expected retention time of 17α-boldenone in a number of the other samples analysed. DARD reported two of these to exceed 0.5ngml^{-1}. However, the presence of 17α-boldenone in these samples was not supported by the results for the qualifier ion transition, m/z 287>135. Small peaks were also observed close to the expected retention time of 17β-boldenone; the highest reported concentration was 0.2ngml^{-1} and, again, the presence of 17β-boldenone in these samples was not supported by the results for the qualifier ion transition.

Investigation of 17α-boldenone and 17β-boldenone analogues

Analysis of substances with similar structures to 17α-boldenone and 17β-boldenone was carried out. Androstenedione eluted between 17β-boldenone and 17α-boldenone and was detected using the MRM transitions employed for 17α- and 17β -boldenone. However, sufficient separation between all of the analytes existed such that androstenedione did not cause interference with the detection of either 17α-boldenone or 17β-boldenone. None of the other compounds investigated caused any analytical interference with the detection and quantification of 17α-boldenone or 17β-boldenone.

Working Range and Calibration Curves

Using the ratio of the analyte (17α-boldenone and 17β-boldenone) peak areas to internal marker ($^2H_3$-17β-boldenone) peak areas gave good linear responses over the calibration range 0.5 to 4ngml^{-1}. Linearity was slightly better for 17β-boldenone than for 17α-boldenone, assumed to be a result of the internal marker having the same stereochemistry as 17β-boldenone. A significant issue in the API LC-MS analysis of samples obtained from biological samples is the potential for ion suppression within the ion source due to co-eluting material, the use of a deuterated analogue minimises such effects as it co-elutes with the analyte and is subject to the same level of ion suppression. A structural analogue also effectively accounts for losses in absolute recovery of the analyte. While

these effects might explain the improved performance of the assay for 17β-boldenone compared to 17α-boldenone the possibility that other factors, such as the presence of low and variable amounts of 17α-boldenone cannot be entirely dismissed. High recovery, good precision and good linearity were obtained over this range with correlation coefficients of 0.991 for 17α-boldenone and 0.996 for 17β-boldenone for the calibration curves examined. The set of quality control standards (QCs) were run alongside in duplicate at low (1ngml^{-1}), medium (2ngml^{-1}) and high (3ngml^{-1}) concentrations. All QC samples analysed were within limits of +/-15%.

Recovery

A summary of the percentage mean recovery results, determined from the back-calculated concentration of the analytes established using a deuterated 17β-boldenone internal standard, from the fortified blank replicates at 1, 1.5 and 2 times the required performance limit are given in the full report submitted to DEFRA. In the interest of brevity only the results for the reporting level (2ngml^{-1}) are presented here in Table 1. All data from the participating laboratories percentage mean recoveries for 17β-boldenone were significantly below the expected value but, with one exception, within the SANCO allowed range of -30% to +10% with an average recovery of 81%, this underestimate was consistent across all the concentrations analysed. Given that the methodology employed a deuterated internal standard it was our expectation that, while results obtained from individual laboratories might fall within the SANCO allowed range the reported values would lie close to, or straddle, the nominal value. This may suggest that a systematic weighing or dilution error was introduced at some point in the production of the 17β-boldenone standard used to prepare the calibration curve or the validation samples. However, careful attention was paid throughout to ensure all laboratories used the same primary standards and that control of weighings and dilutions was maintained and the possibility that other factors contributed to the consistently low recovery of 17β-boldenone cannot be discarded.

The recovery of 17α-boldenone was variable and on 5 of the 9 occasions outside the range allowed by SANCO. Again this reflects the fact that the assay performance for 17α-boldenone was less than that of 17β-boldenone.

	Laboratory		
Batch	**DARD**	**HFL**	**LGC**
% Mean Recovery of 17α-boldenone (2SF)			
1	120	150	81
2	110	87	100
3	110	140	97
% Mean Recovery of 17β-boldenone (2SF)			
1	84	85	60
2	85	75	79
3	86	91	89

Table 1: Recovery of 17α boldenone and 17β-boldenone from bovine urine fortified at 2ngml^{-1}

Repeatability

A summary of the repeatability results from the fortified blank replicates at 1, 1.5 and 2 times the required performance limit is given in the full report submitted to DEFRA. In the interest of brevity the only the results for the reporting level (2ngml^{-1}) are presented here in Table 2.

	Laboratory		
	DARD	**HFL**	**LGC**
Repeatability of 17α-boldenone determination (2SF)			
Mean (ngml^{-1})	2.3	2.5	1.9
%CV	14	35	28
Repeatability of 17β-boldenone determination (2SF)			
Mean (ngml^{-1})	1.7	1.7	1.5
%CV	4.4	11	24

Table 2: Repeatability of 17α boldenone and 17β -boldenone determinations from bovine urine fortified at 2ngml^{-1}

The mean values for recovered 17β-boldenone are all below the fortified levels. Repeatability (expressed in terms of %CV) for 17α-boldenone and 17β-boldenone at all levels ranged between 14% and 35% and between 4.4% and 28%, respectively. The slightly better repeatability for 17β-boldenone is attributed to the fact the internal standard is a deuterated analogue of this analyte.

Reproducibility

Six batches of replicates were analysed, three from each laboratory (HFL and LGC). The three batches with the highest %CV's and %RE's for 17β-boldenone at the fortification level of 2ngml^{-1} were chosen to complete the calculation for the reproducibility of the assay. Hence, HFL Batch 2, LGC Batch 1 and 2 were chosen as the worst-case examples for this purpose. The results are presented in Table 3.

A summary of the reproducibility results from the fortified blank replicates at 1, 1.5 and 2 times the required performance limit are given in the full report submitted to DEFRA.

Laboratory		17α-boldenone	17β-boldenone
HFL (B2) + LGC (B1+B2)	Mean	1.8ngml^{-1}	1.4ngml^{-1}
	%CV	30%	22%
	%RE	-10%	-29%

Table 3: Reproducibility of 17α- and 17β-boldenone determinations in bovine urine fortified at 2ngml^{-1} (worst-case batches) (to 2SF)

Data from DARD was not used to calculate the combined reproducibility because the DARD laboratory, in this instance, was used as the control laboratory. DARD's methodology for sample extraction and analysis differs slightly to the other two participating laboratories.

The best-case scenario for the reproducibility of 17α- and17β-boldenone at the fortification level of 2ngml^{-1} was also calculated using the data from the remaining batches, these results are presented in Table 4. Again, in both instances, the level of 17β-boldenone was under estimated. The reproducibility of the results for 17β-boldenone were significantly better than those for 17α-boldenone, again attributed to the fact that the internal standard was an analogue of 17β-boldenone.

Laboratory		17α-boldenone	17β-boldenone
HFL (B1 +3) + LGC (B3)	Mean	2.5ngml^{-1}	1.8ngml^{-1}
	%CV	31%	6.6%
	%RE	27%	-12%

Table 4: Reproducibility of 17α-boldenone and 17β-boldenone determinations in bovine urine fortified at 2ngml^{-1} (best-case batches) (to 2SF)

Decision Limit (CCα)

The decision limits for each of the participating laboratories at the required performance limit have been calculated using the back-calculated concentrations of the analytes. Data are presented in Table 5.

Laboratory	CCα 17α-boldenone	CCα 17β-boldenone
DARD	0.8ngml^{-1}	0.2ngml^{-1}
HFL	1.0ngml^{-1}	0.5ngml^{-1}
LGC	1.0ngml^{-1}	1.0ngml^{-1}

Table 5: CCα values for each analyte and laboratory

The results show that for 17α-boldenone with an MRPL of 1ngml^{-1} the methodology is operating near the limit of its capability. For 17β-boldenone the capability of the assay appears adequate for an MRPL of 1ngml^{-1}, again this improved performance for 17β-boldenone is largely attributed to the internal standard. Whist it is desirable that the assay performs well at the 1ngml^{-1} level for both analytes, this is not essential for success. The reported data suggest that the levels of 17α-boldenone produced by faecal contamination are significantly higher than those for 17β-boldenone and that presence of the latter is much more indicative of a case of abuse of boldenone, its esters or precursors.

CONCLUSIONS

An LC-MS/MS methodology for screening bovine urine based upon a generic steroid screen used in the residue testing laboratory at LGC has been optimised to screen for the presence of 17α and 17β-boldenone. The method has been evaluated across three laboratories according to the guidelines contained in SANCO/1085/2000 Revision 6.

The ruggedness of the proposed methodology was tested prior to the full screening method validation using a Youden approach. No significant factors affecting the performance of the assay were identified.

The stability of 17α-boldenone and 17β-boldenone was investigated. Both 17α-boldenone and 17β-boldenone were found to be stable in the final analysis solution for at least one week and at least 20 weeks in the fortification solution used to prepare calibration and quality control samples.

The results obtained from the evaluation indicate that the proposed screening methodology is adequately robust for its intended use. This conclusion is based upon the methodology demonstrating applicability, ruggedness and robustness for the analysis of 17α-boldenone and 17β-boldenone at the reporting level of 2ngml^{-1}. The methodology appears to be reaching the limits of its capability for 17α-boldenone at about 1ngml^{-1}. This is not considered a major issue as current knowledge indicates that the presence of 17β-boldenone is much more indicative of potential abuse of boldenone, its esters or precursors.

APPENDIX 1

METHOD SOP

1 Title

Validation of a LC-MS/MS Multi-Residue Screening Method for the Analysis of 17α- and 17β-Boldenone in Bovine Urine

Warning – Persons using this International Standard should be familiar with normal laboratory practice. This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

2 Scope

This is a liquid chromatographic-mass spectroscopy (LC-MS) assay for the screening analysis of 17α- and 17β-boldenone in bovine urine, with a minimum required performance limit of 1ngml^{-1}.

From research there are no critical factors that should be particularly noted to ensure the ruggedness of this methodology. Although one should note that during solid phase extraction the cartridges should never be subjected to full vacuum (drying a cartridge is the exception to this).

3 Principle

The screening procedure utilises 5ml of bovine urine which is initially subjected to enzyme hydrolysis followed by a solid phase extraction (SPE) clean-up using two different sorbents, C18 and NH_2 phase cartridges. The eluent is then evaporated and reconstituted in the reconstitution solvent. Aliquots (20µl) are analysed using a LC-MS/MS system in positive electrospray mode under MRM conditions, acquiring the transitions 290>138, 290>121, 287>135 and 287>121.

4 Reagents and Materials

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and deionised water.

4.1 Reagents

Sodium acetate buffer (2M, pH5.0)
Helix pomatia digestive juice (131units/µl)
Methanol
Water:methanol (80:20)
Ethyl acetate:methanol (60:40)
Ammonium formate buffer (25mM pH4.5)

4.2 Materials

Bakerbond C18 e/c 500mg 3ml cartridges, Part no: 7020-03
Phenomenex Strata amine 500mg cartridges, Part no: 8B-S009-HBJ
Phenomenex Luna C18(2), 150 x 2.0mm, 3µm, Part no: 00F-4251-B0
Phenomenex security guard column system.Holder, 4.0 L mm, Part no.:KJO-4282, cartridge C18 4.0 L mm x 3.0 ID mm, Part no: AJO-428

5 Apparatus

Liquid-chromatographic-mass spectrometric/mass spectrometric system (LC-MS/MS)
(Specific system used: Waters 1525µ Binary HPLC pump attached to a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer)
Bench-top centrifuge, capable of accepting 20ml glass scintillation screw cap vials
Heating block at 70^{0}C, capable of accepting 3ml vials, 5ml and 10ml glass tubes
Bench-top vortex mixer
Pipette 5ml, air displacement
Pipette 50µl, positive displacement

Pipette 250µl, positive displacement

6 Sampling

Laboratory samples, known as the test sample should be stored at -20°C or lower until needed for analysis.

7 Drug Standards

7.1 Stock Solutions

17β-boldenone standard solution: 1mgml^{-1} in methanol
17α-boldenone standard solution: 100µgml^{-1} in methanol
$^{2}H_3$-17β-boldenone internal marker: nominal 1mgml^{-1} in methanol

7.2 Reference Solutions

17β-boldenone standard solution: 20µgml^{-1} in methanol
17α-boldenone standard solution: 20µgml^{-1} in methanol
$^{2}H_3$-17β-boldenone internal marker: nominal 10µgml^{-1} in methanol

8 Procedure

8.1 Sample Preparation

NB: If test sample has been frozen, defrost at room temperature.

Prepare a mixed internal marker working solution by pipetting the volumes of drug reference solutions detailed in Table 1 into a 10ml volumetric flask and make up to the mark with methanol.

Drug	Ref. Solution Conc.	Vol. Working Soln.
$^{2}H_3$-17β-boldenone	10µgml^{-1}	100µl

Table 1: Preparation of internal marker working solution

8.1.1 Remove a 5ml portion of the test sample to a 20ml capped plastic vial
8.1.2 Add 50µl internal marker of working solution to the test sample and mix
8.1.3 Spike sample with 17α- and/or 17β-boldenone at appropriate levels as required
8.1.4 Add 2ml sodium acetate buffer
8.1.5 Check the pH manually and add more buffer if needed to bring the pH of the urine to pH 5
8.1.6 Add 50µl helix pomatia, enzyme to the test sample and mix
8.1.7 Heat for 1 hour in an oven set at 37°C
8.1.8 Centrifuge urine samples for 10 minutes @ 2000rpm prior to extraction

8.2 Preparation of Calibration Line

NB: Calibration line and controls must be prepared by different analysts.

Pipette the following volumes of drug standards into a 10ml volumetric flask (see Table 2) and make up to the mark with methanol. One mixed standard is known as A1 the other is known as A2.

Drug	Ref solution concentration	Volume
17α-boldenone	20µgml^{-1}	50µl
17β-boldenone	20µgml^{-1}	50µl

Table 2: Preparation of mixed standard working solution.

8.2.1 Remove a 5ml portion of the test sample to a 20ml capped plastic vial
8.2.2 Add 50µl internal marker of working solution to the test sample and mix as 8.1.2
8.2.3 Spike sample with A1 as shown in Table 3

STD/ Calibration level		Vol of A1 (µl)	Equivalent Conc. in Urine ($ngml^{-1}$)	
			17α-boldenone	17β-boldenone
BLANK	A	0	0	0
	B			
1(1/4 mprl*)	A	25	0.5	0.5
	B			
2 (1/2mprl)	A	50	1	1
	B			
3 (mprl)	A	100	2	2
	B			
4 (1.5mprl)	A	150	3	3
	B			
5 (2mprl)	A	200	4	4
	B			

Table 3: Preparation of calibration line.

8.2.4 Follow steps 8.1.4 to 8.1.8

8.3 Preparation of Quality Controls

8.3.1 Dispense six 5ml portions of blank urine
8.3.2 Add 50µl internal marker of working solution to the test sample and mix as 8.1.2
8.3.3 Spike sample with A2 as shown in Table 4

Control		Vol of A2 (µl)	Equivalent Conc in Urine ($ngml^{-1}$)	
			17α-boldenone	17β-boldenone
Low	A	50	1	1
	B			
Medium	A	100	2	2
	B			
High	A	150	3	3
	B			

Table 4: Preparation of calibration line

8.3.4 Follow steps 8.1.4 to 81.8

8.4 Extraction

SPE Conditions:

8.4.1 C18 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml of water
Load the hydrolysed urine sample extract onto the cartridge
Wash the cartridge with 5ml of water
Wash the cartridge with 5ml of water:methanol (80:20 v/v)
Dry cartridge for approximately 15 minutes using the vacuum manifold
Elute from the C18 cartridge with 5ml of ethyl acetate:methanol (60:40v/v)

8.4.2 NH_2 cartridge: Condition cartridge with 5ml of methanol
Condition cartridge with 5ml ethyl acetate : methanol (60:40 v/v)
Load sample extract from C18 cartridge in step 8.2.1 and collect eluate

8.4.3 Evaporate the eluate to dryness under Oxygen Free Nitrogen (OFN) on a heating block at 70°C
8.4.4 Reconstitute the dried extract in 350µl of 50:50 water:methanol

8.4.5 Whirlimix, transfer to a labelled LC-MS vial and cap
8.4.6 Analyse the samples by LC-MS/MS

8.5 Preparation of a System Suitability Standard

8.5.1 Pipette 50µl of 17α- and 17β-boldenone and 50µl of internal marker (D3-boldenone) into a 3ml glass vial
8.5.2 Evaporate the solvent under OFN on a heating block at 70°C
8.5.3 Reconstitute the dried extract in 350µl of 50:50 water:methanol
8.5.4 Transfer to a labelled LC-MS vial, cap and submit to LC-MS/MS

9 Analysis

9.1 LC-MS Conditions

Autosampler instrument: Waters 2777 Sample Manager
Pump: 1525µ binary HPLC pump and column oven
Software: MassLynx 4.0 SP2

9.2 LC Conditions

LC instrument: Waters 1525µ Binary HPLC pump
Column: Phenomenex Luna C18(2), 150 x 2.0mm, 5µm; Part No: 00F-4252-B0
Guard Column: Phenomenex security guard column system.
Holder, 4.0 L mm, Part no.: KJO-4282
Cartridge C18 4.0 L mm x 3.0 ID mm, Part no: AJO-4287
Column temp.: Ambient
Mobile phase: Solvent A: Ammonium formate, 25mM, pH4.5
Solvent B: Methanol
Injection volume: 20µl
Run time: 22 minutes

Time (min)	% Solvent A	% Solvent B	Flow (ml/min)
0	40	60	0.2
1	40	60	0.2
15	10	90	0.2
16	10	90	0.2
16.5	40	60	0.2
22	40	60	0.2

Table 5: Gradient elution timetable (linear gradient)

9.3 Mass Spectrometer Conditions

MS instrument: Waters Micromass Quattro Premier tandem quadrupole mass spectrometer or equivalent. Note: The optimum conditions for the generation and transmission of ions will vary somewhat from instrument to instrument. Therefore, these values should be used only as a guide.

Divert Valve:

Time (min)	0.00 - 6.00	6.00 - 16.00	16.00 - 22.00
Position	Waste	Detector	Waste

Table 6: Divert valve conditions

Source settings: **ESI+**
Capillary (kV): 3.50
RF Lens 1: 33
Aperture: 0.2
RF Lens 2 0.2
Source temp: 120°C
Desolvation temp: 450°C
Cone gas flow: Approximately 90 L/Hr Nitrogen

Desolvation gas flow: Approximately 500 L/Hr Nitrogen

Precursor Ion (Da)	Product Ion (Da)	Dwell (sec)	Cone (V)	Collision (eV)
271.20	199.20	0.2	20	18
271.20	253.20	0.2	20	15
275.20	109.03	0.2	20	16
275.20	145.07	0.2	20	16
278.30	145.09	0.2	30	22
278.30	148.10	0.2	30	20
287.25	121.00	0.2	20	22
287.25	135.05	0.2	20	14
290.10	121.00	0.2	35	25
290.10	137.90	0.2	35	15
303.10	97.00	0.2	20	15
303.10	109.00	0.2	20	18
306.20	108.90	0.2	40	20

Table 7: MRM of 13 mass pairs, Time 0.5 to 15 min, ESI+.

10 Data Interpretation

17α- and 17β-boldenone and their internal marker, $^{2}H_{3}$-17β-boldenone are detected by LC-MS/MS. The expected retention times and monitored ions are detailed in Table 8. $^{2}H_{3}$-17β-boldenone should be indicated in all test portions by the presence of a peak at the correct retention time and by the required ion transitions being present under positive ESI conditions.

Drug	RT (min)	RRT	Precursor ion	Product ions
$^{2}H_{3}$-boldenone (IM)	9.90	1	290	138, 121
17β-boldenone	9.95	1.01	287	135, 121
17α-boldenone	11.92	1.22	287	135, 121

Table 8: Retention times and monitored ions of analytes.

For confirmatory purposes, when MRM analysis is employed, four identification points must be secured. These comprise the analyte RT and three mass spectral ions (one precursor ion and two daughter ions). The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion or transition, must correspond to those of the suitability standard with the tolerances shown in Table 9.

Relative Intensity (% of base peak)	LCMS Tolerance (relative)
>50%	+/- 20%
>20% - 50%	+/- 25%
>10% - 20%	+/- 30%
</=10%	+/- 50%

Table 9: Maximum permitted tolerances for relative ion intensities using a mass spectrometric technique

Interfering ions in the spectra should be less than 20% of the intensity of the most intense ion

10.1 Integrate and ratio the areas of the analyte and internal marker in extracted curve and samples for determination. The ion ratios obtained in samples for determination should correspond to those obtained from the extracted curve samples.

10.2 Plot the calculated ratios against spiked concentration. The value for a sample for determination can then be obtained from the curve.

10.3 Ensure that the ratios of the chromatographic retention times of 17α-boldenone and 17β-boldenone to that of the internal standard, ie the relative retention times (RRTs) obtained for the samples for determination are within +/- 0.5% of those obtained for the extracted sample and system suitability standard.

10.4 Any ratio of analyte:internal marker obtained from a sample for determination that is at or above the value obtained for an extracted standard of concentration 0.5 x MRPL should be submitted for confirmatory analysis.

Appendix 2

METHOD VALIDATION PLAN

The method will be validated in the range of 0.5ng/ml to 4ng/ml for 17α- and 17β-boldenone. It is intended to demonstrate adequate capability at a concentration of 1ngml^{-1} in urine.

At HFL, analysis will be carried out using a Waters Quattro Premier series, triple quadrupole LCMS system or equivalent. Analysis of samples by LGC and DARD will be analysed using systems employed for the statutory testing programme.

Assay validation will comprise assessment of the following:

- Applicability/Ruggedness
- Stability
- Selectivity/Specificity
- Working range and calibration curve
- Trueness/Recovery
- Repeatability
- Reproducibility
- Decision limit

All positive identifications of 17α- or 17β-boldenone must satisfy the following criteria:

The ratio of the chromatographic retention time of the analyte to that of the internal standard, ie the relative retention time (RRT), must correspond to that of the system suitability standard at a tolerance of +/- 2.5%.

The SANCO guidelines covering screening methods allow for just one SRM transition to be used for quantification if another separate transition is used for qualification. Diagnostic ions are required to have signal to noise ratios of >3:1.

Applicability/Ruggedness (minor changes)

Ruggedness testing has been performed prior to validation. Minor changes in the analysis were deliberately introduced and their consequences upon the quantification and detection of 17α- and 17β-boldenone at the 2ngml^{-1} level determined. A Youden approach was applied to key elements from the pre-treatment, clean-up and analysis phases of the method, thereby demonstrating the overall applicability and ruggedness of the methodology.

Stability of 17α- and 17β-boldenone

The stability of 17α- and 17β-boldenone in the solvent used for fortification and in final reconstitution solvent will also be investigated under the following conditions during validation of the screening method:

1) in the dark at -20ºC
2) in the dark at +4ºC
3) in the dark at room temperature
4) under normal lighting conditions at room temperature

Analysis will be carried out at 0,1,2,3,4 and 20 weeks or until degradation phenomena are observable.

Specificity

An evaluation of specificity will include two parts.

Firstly, an evaluation of a limited number of substances that might give rise to an interfering signal will be carried out. Compounds with similar structures or molecular weights and substances likely to be used in combination with the analyte will be examined, for example other steroids, both exogenous and endogenous. Other steroids will include testosterone, nandrolone, androstenedione, etc. The assessment will be carried out by comparison of

chromatograms for the presence of peaks likely to interfere with analysis of 17α- and 17β-boldenone or the internal marker.

Secondly, potential interferences from the sample matrix will be investigated. This will be achieved by the analysis, at each laboratory, of a minimum of 20 blank bovine samples taken from animals not known to have been administered any drug substances. The samples will be fortified with internal marker alone and then analysed for any interferences in the region of the chromatogram where 17α- and 17β-boldenone elute. Additional chromatographic peaks in the region of the analyte(s) under investigation will be further assessed for: 1) the possibility of producing false positives; 2) any masking of responses that may lead to false negatives; 3) any influence upon quantification.

The chromatographic system will be checked for injection carry-over.

Working range and calibration curve

At least two independent sets of analyte standards at 0, 0.5, 1, 2, 3 and 4ngml^{-1}will be prepared and analysed.

All subsequent quantitative work will be carried out using appropriate standard curves using a minimum of 5 levels, ie 0.5,1,2,3 and 4ngml^{-1} in duplicate. The analytical result should fall within the range of the curve.

Recovery

Recovery will be determined by analysis of 6 fortified blank replicates each at 1.0, 1.5 and 2ngml^{-1}. The percentage recovery, standard deviation and coefficient of variation will be determined at each level.

Repeatability

Repeatability will be determined by three separate laboratories. Each laboratory will analysis 6 fortified blank replicates each at 1.0, 1.5 and 2ngml^{-1} on three separate occasions (54 samples in total in each participating laboratory). The mean concentration, standard deviation and coefficient of variation within each laboratory will be determined at each level and on each occasion.

Reproducibility

Reproducibility will be determined by analysis of 6 fortified blank replicates each at 1.0, 1.5 and 2ngml^{-1} on three separate occasions at the three separate laboratories. Therefore, the conditions are varied (ie different analyst). The data collected from the repeatability test will be used for calculating the reproducibility of the method. The overall mean concentration, standard deviation and coefficient of variation will be determined at each level and on each occasion.

Decision Limit (CCα)

The decision limit will be determined by the calibration curve procedure as outlined in the SANCO guidelines. Data collected from the repeatability determinations will be used for this purpose. The corresponding concentration at the y-intercept plus 1.64 times the standard deviation of the reproducibility of the intercept will provide a value for the decision limit (α - 5%).

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.

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